01 JUN 1998

FORM PTO-1390 (REV. 01-98) U.S. DEPARTMENT OF COMMERCE PATENT AND TR

TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371

ATTORNEY'S DOCKET NUMBER	U.S. APPLICATION NO. (If known, see 37 CFR 1.5)
875.001US2	09/077572

INTERNATIONAL APPLICATION NO.	INTERNATIONAL FILING DATE	PRIORITY DATE CLAIMED
PCT/US96/18984	27 November 1996 (27.11.96)	01 December 1995 (01.12.95)

TITLE OF INVENTION NON-TOXIC MUTANTS OF PATHOGENIC GRAM-NEGATIVE BACTERIA

APPLICANT(S) FOR DO/EO/US APICELLA, Michael A. et al.

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. ☒ This is a **FIRST** submission of items concerning a filing under 35 U.S.C. 371.
2. ☐ This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S.C. 371.
3. ☒ This express request to begin national examination procedures (35 U.S.C. 371(f)) at any time rather than delay examination until the expiration of the applicable time limit set in 35 U.S.C. 371(b) and PCT Articles 22 and 39(1).
4. ☒ A proper Demand for International Preliminary Examination was made by the 19th month from the earliest claimed priority date.
5. ☒ A copy of the International Application as filed (35 U.S.C. 371(c)(2))
   a. ☐ is transmitted herewith (required only if not transmitted by the International Bureau).
   b. ☒ has been transmitted by the International Bureau.
   c. ☐ is not required, as the application was filed in the United States Receiving Office (RO/US).
6. ☐ A translation of the International Application into English (35 U.S.C. 371(c)(2)).
7. ☒ Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371(c)(3))
   a. ☐ are transmitted herewith (required only if not transmitted by the International Bureau).
   b. ☐ have been transmitted by the International Bureau.
   c. ☐ have not been made; however, the time limit for making such amendments has NOT expired.
   d. ☒ have not been made and will not be made.
8. ☐ A translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)).
9. ☐ An oath or declaration of the inventor(s) (35 U.S.C. 371(c)(4)).
10. ☐ A translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371(c)(5)).

Items 11. to 16. below concern document(s) or information included:

11. ☐ An Information Disclosure Statement under 37 CFR 1.97 and 1.98.
12. ☐ An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.
13. ☒ A FIRST preliminary amendment.
    ☐ A SECOND or SUBSEQUENT preliminary amendment.
14. ☐ A substitute specification.
15. ☐ A change of power of attorney and/or address letter.
16. ☒ Other items or information:

"Express Mail" mailing label number: EM492672567US

Date of Deposit: 01 June 1998

I hereby certify that this paper or fee is being deposited with the United States Postal Service "Express Mail Post Office to Addressee" service under 37 CFR 1.10 on the date indicated above and is addressed to the Assistant Commissioner for Patents, Washington, D.C. 20231

Printed Name Sarah Gowdy

Signature Sarah L Gowdy

Copies of: Published International Application No. WO 97/19688, which includes specification, claims, abstract, drawings and international search report; International Preliminary Examination Report with attached claim replacement page 69; Chapter II Demand for Examination; Notification of Receipt of Demand; PCT Request Form.

Check for filing fee - $1562.00 and a return postcard.

U.S. APPLICATION NO. (if known, see 37 CFR 1.5)	INTERNATIONAL APPLICATION NO	
	PCT/US96/18984	875.001US2

17. ☒ The following fees are submitted:

BASIC NATIONAL FEE (37 CFR 1.492 (a) (1) - (5)) :

	CALCULATIONS PTO USE ONLY
Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO and International Search Report not prepared by the EPO or JPO **$1070.00**	
International preliminary examination fee (37 CFR 1.482) not paid to USPTO but International Search Report prepared by the EPO or JPO **$930.00**	
International preliminary examination fee (37 CFR 1.482) not paid to USPTO but international search fee (37 CFR 1.445(a)(2)) paid to USPTO **$790.00**	
International preliminary examination fee paid to USPTO (37 CFR 1.482) but all claims did not satisfy provisions of PCT Article 33(1)-(4) **$720.00**	
International preliminary examination fee paid to USPTO (37 CFR 1.482) and all claims satisfied provisions of PCT Article 33(1)-(4) **$98.00**	
ENTER APPROPRIATE BASIC FEE AMOUNT =	$ 1070.00
Surcharge of **$130.00** for furnishing the oath or declaration later than ☐ 20 ☒ 30 months from the earliest claimed priority date (37 CFR 1.492(e)).	$ 130.00

CLAIMS	NUMBER FILED		NUMBER EXTRA	RATE	
Total claims	29	- 20 =	9	X **$22.00**	$ 198.00
Independent claims	5	- 3 =	2	X **$82.00**	$ 164.00
MULTIPLE DEPENDENT CLAIM(S) (if applicable)				+ **$270.00**	-0-
TOTAL OF ABOVE CALCULATIONS =					$ 1562.00
Reduction of 1/2 for filing by small entity, if applicable. A Small Entity Statement must also by filed (Note 37 CFR 1.9, 1.27, 1.28).					$ ---
SUBTOTAL =					$ 1562.00
Processing fee of **$130.00** for furnishing the English translation later than ☐ 20 ☐ 30 months from the earliest claimed priority date (37 CFR 1.492(f)). +					$ ---
TOTAL NATIONAL FEE =					$ 1562.00
Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31). **$40.00** per property +					$ ---
TOTAL FEES ENCLOSED =					$ 1562.00
					Amount to be: refunded $
					charged $

a. ☒ A check in the amount of $ 1562.00 to cover the above fees is enclosed.

b. ☐ Please charge my Deposit Account No. _____ in the amount of $_____ to cover the above fees. A duplicate copy of this sheet is enclosed.

c. ☒ The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. 19-0743. A duplicate copy of this sheet is enclosed.

NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b)) must be filed and granted to restore the application to pending status.

SEND ALL CORRESPONDENCE TO:

Ann S. Viksnins
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SIGNATURE:

Ann s. Viksnins
NAME

37,748
REGISTRATION NUMBER

09/077572

01 JUN 1998

PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant:	APICELLA, Michael A. et al.	Examiner: Unknown
Serial No.:	Unknown	Group Art Unit: Unknown
Filed:	Concurrently herewith	Docket: 875.001US2
Title:	NON-TOXIC MUTANTS OF PATHOGENIC GRAM-NEGATIVE BACTERIA	

PRELIMINARY AMENDMENT

BOX PCT
Assistant Commissioner for Patents
Washington, D.C. 20231

Sir:

Before taking up the above-identified U.S. national phase patent application for examination, please enter the replacement claim page 69 that is attached to the International Preliminary Examination Report dated 15 April 1998 for corresponding claim page 69 as originally filed.

Please enter the above-described amendment before taking up the application for examination.

Respectfully submitted,

APICELLA, Michael A. et al.

By Applicants' Attorneys,

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Date: 01 June 1998
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By _____
Ann S. Viksnins
Reg. No. 37,748

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Printed Name Sarah L. Goudy

Signature _____

NON-TOXIC MUTANTS OF PATHOGENIC GRAM-NEGATIVE BACTERIA

This invention has been made with government support under grant AI 24616 awarded by the National Institutes of Health. The government has certain rights in the invention.

Field of the Invention

The present invention relates to compositions comprising altered endotoxin (lipooligosaccharide (LOS); lipopolysaccharide (LPS)) of gram-negative bacterial pathogens. More particularly, the present invention relates to the making of a form of endotoxin, by a genetically engineered gram-negative pathogen, which lacks a substantially toxic lipid A portion. Also disclosed are prophylactic and therapeutic uses of the substantially detoxified endotoxin, and of mutant gram-negative bacteria producing the substantially detoxified endotoxin.

Background of the Invention

Gram-negative bacteria have an outer membrane comprised of components including proteins, lipoproteins, phospholipids, and glycolipids. The glycolipids comprise primarily endotoxin-lipopoly-saccharides (LPS) or lipooligosaccharides (LOS), depending on the genus of bacteria. LPS are molecules comprised of

a) a lipid A portion which consists of a glucosamine disaccharide that is substituted with phosphate groups and long chain fatty acids in ester and amide linkages;
b) a core polysaccharide which is attached to lipid A by an eight carbon sugar, KDO (ketodeoxyoctonoate), and heptose, glucose, galactose, and N-acetylglucosamine; and

c) an O-specific side chain comprised of repeating oligo-saccharide units which, depending on the genera and species of bacteria, may contain mannose, galactose, D-glucose, N-acetylgalactosamine, N-acetylglucosamine, L-rhamnose, and a dideoxyhexose (abequose, colitose, tyvelose, paratose, trehalose). LOS has a similar structure as LPS, containing a lipid A portion and a complex carbohydrate structure, but differs in that it does not contain repeating O-side chains.

The major antigenic determinants of gram-negative bacteria are believed to reside in the carbohydrate structure of the O-specific side chain of LPS and the complex carbohydrate structure of LOS. These carbohydrate structures may vary for different species of the same genera of gram-negative bacteria by varying one or more of the sugar composition; the sequence of oligosaccharides; the linkage between the oligosaccharides; and substitutions/modifications of an oligosaccharide (particularly a terminal oligosaccharide).

LPS and LOS have been considered as bacterial components which have potential as vaccine immunogens because of the antigenic determinants ("epitopes") residing in their carbohydrate structures. However, the chemical nature of LPS and LOS prevent the use of these molecules in vaccine formulations; i.e., active immunization with LPS or LOS is unacceptable due to the inherent toxicity of the lipid A portion. The patho-physiologic effects induced (directly or indirectly) by lipid A of LPS or LOS in the bloodstream include fever; leucopenia; leucocytosis; the Shwartzman reaction; disseminated intravascular coagulation; abortion; and in larger doses, shock and death. Accordingly, there are no currently available vaccines which induce antibody responses to LPS or LOS epitopes.

As shown in FIG. 1, the lipid A portion of endotoxin generally comprises a hydrophilic backbone of glucosamine disaccharide which is either monophosphorylated or diphosphorylated (positions 1 and 4'); and which carries at least six molecules of ester- and amide-bound fatty acids. Four molecules of (R)-3-hydroxytetradecanoate (e.g. 3-hydroxy-myristoyl or β-hyroxymyristic acid or β-OH) are linked directly to the lipid A backbone at positions 2, 3, 2', and 3'. Hydroxyl groups of two of the four molecules of β-OH are substituted with normal fatty acids (termed "secondary acyl chains", and including dodecanoate, tetradecanoate, and hexadecanoate) in forming acyloxyacyl groups.

One approach to making a detoxified endotoxin molecule involves isolating the endotoxin, and enzymatically-treating the isolated endotoxin with a human neutrophilic acyloxyacyl hydrolase (U.S. Patent Nos. 4,929,604, 5,013,661 and 5,200,184). The acyloxyacyl hydrolase hydrolyzes the fatty acids (non-hydroxylated, secondary acyl chains) from their ester linkages to hydroxy groups of β-OH (hydroxylated). The resultant altered endotoxin, from enzymatic treatment, contained a lipid A moiety lacking non-hydroxylated fatty acids. This altered endotoxin exhibited reduced *in vivo* toxicity, but retained antigenicity.

Another approach involves a method of modifying isolated endotoxin by selectively removing the β-OH that is ester-linked to the reducing-end glucosamine backbone at position 3 (U.S. Patent No. 4,912,094; Reexamination B1 4,912,094). The selective removal of β-OH was accomplished using alkaline hydrolysis. The resultant modified endotoxin exhibited reduced *in vivo* toxicity, but retained antigenicity.

Both approaches involve chemically treating isolated endotoxin. Neither approach discloses the production in a gram negative bacterial pathogen of an

endotoxin having substantially reduced toxicity, yet retaining antigenicity. Further, there has been no disclosure of the use of a gram-negative bacteria, which has been engineered to produce an endotoxin having substantially reduced toxicity and yet retaining antigenicity, in a prophylactic or therapeutic vaccine against endotoxic shock and gram-negative bacteremia.

Summary of the Invention

The present invention is directed to a method for producing, in a mutant gram-negative pathogen, LPS or LOS which exhibits substantially reduced toxicity as compared to the wild type endotoxin, and which retains the antigenicity of its corresponding wild type endotoxin. The method comprises creating a mutation in the *htrB* gene of the gram-negative bacterial pathogen such that there is a lack of functional HtrB protein in the mutated gram-negative bacterial pathogen. It was found that lipid A produced by the *htrB* mutant lacks one or both of the fatty acids (non-hydroxylated or secondary acyl chains) thereby rendering the endotoxin in an isolated form, or the mutant gram-negative bacterial pathogen bearing the endotoxin, substantially reduced in toxicity and yet retaining antigenicity, as compared to wild type. Endotoxin isolated from *htrB* mutants, or the *htrB* mutants themselves (whole cell vaccine), can be used to immunize individuals at risk of gram-negative bacteremia by inducing antibodies to the major antigenic determinants which reside in the carbohydrate structure of the O-specific side chain of LPS and the complex carbohydrate structure of LOS. Further, the *htrB* mutants can be engineered to express heterologous antigens of other microbial pathogens at the surface of the *htrB* mutant for presentation to a vaccinated individual's immune system in a multivalent vaccine. Also, the endotoxin isolated from the *htrB*

mutants of the present invention may be used to generate LPS or LOS-specific antibody which may be useful for passive immunization and as reagents for diagnostic assays directed to detecting the presence of gram-negative bacterial pathogens in clinical specimens.

Brief Description of the Figures

FIG. 1 is a schematic representation of the general structure of lipid A of gram negative bacteria of the family *Enterobacteriaceae*.

FIG. 2A is a schematic representation of the general structure of a species of lipid A, from the LOS of an *htrB* mutant, comprising pentaacyl diphosporyl lipid A.

FIG. 2B is a schematic representation of the general structure of a species of lipid A, from the LOS of an *htrB* mutant, comprising tetraacyl diphosporyl lipid A.

FIG. 3 is a graph showing the relative toxicity of an *htrB* mutant (O, Δ) as compared to wild type bacteria (□) in a TNFα release assay.

FIG. 4 is a photograph showing human primary respiratory epithelial cells unstimulated (control), exposed to NTHi 2019 LOS, or exposed to *htrB* mutant B29 LOS, and reacted with either a fluorescent probe that hybridizes to TNFα mRNA (probe 1) or a fluorescent control probe (probe 2).

FIG. 5 is a graph showing mean titers of anti-LOS antibody against NTHI 2019 LOS (antigen coating) in ELISA from mice immunized with NTHi 2019 (Pool 595), *htrB* mutant B29 LOS (Pool 597), or *htrB* mutant B29 LOS conjugated to a carrier protein (Pool 606), with adjuvant.

FIG. 6 is a graph showing the mean titers of anti-LOS antibody against *htrB* mutant B29 LOS (antigen coating) in ELISA from mice immunized with NTHi 2019 (Pool 595), *htrB* mutant B29 LOS (Pool 597), or *htrB* mutant B29 LOS conjugated to a carrier protein (Pool 606), with adjuvant.

FIG. 7 is a schematic representation comparing the structures of wild type *Salmonella* lipid A and *htrB* mutant lipid A.

Detailed Description of the Invention

Definitions:

"Endotoxin" is a term used herein for purposes of the specification and claims to refer to the LPS or LOS of gram-negative bacterial pathogens, wherein the endotoxin is either in a cell-associated or isolated form. "*htrB* endotoxin" and "*htrB* mutant endotoxin" refer to endotoxin isolated and purified from an gram-negative bacterial pathogen *htrB* mutant.

"vaccine candidate or vaccine antigen" is a term used herein for purposes of the specification and claims to refer to an endotoxin epitope having one or more of the following properties (a-d): (a) is immunogenic; (b) is surface-exposed (which can be shown by techniques known in the art including immunofluorescence assays, electron microscopy staining procedures, and by bactericidal assays); (c) induces antibody having bactericidal activity in the presence of complement and/or functions in immune clearance mechanisms; (d) induces antibody which neutralizes other functional activity of the epitope (immunogenicity, or toxicity, etc.).

"Gram-negative bacterial pathogen" is a term used herein for the purposes of the specification and claims to refer to one or more pathogenic (to humans or animals) bacterium of a genus and species including *Neisseria meningitidis*, *Neisseria gonorrhoeae*, *Haemophilus influenzae*, *Haemophilus ducreyi*, other *Haemophilus* species, *Moraxella catarrhalis*, *Campylobacter jejuni*, *Salmonella typhimurium*, other

Salmonella species, *Shigella dysentariae*, and other *Shigella* species, and *Pseudomonas aeruginosa*.

"Substantially reduced in toxicity" is a term used herein for the purposes of the specification and claims to refer to a reduction in bioactivity of at least 10 fold to 100 fold or more as compared to wild type endotoxin.

"Carrier protein" is a term used herein for the purposes of the specification and claims to refer to a protein which is conjugated to the *htrB* mutant endotoxin. While the *htrB* mutant endotoxin appears to be immunogenic on its own, it is known in the art that conjugation to a carrier protein can facilitate immunogenicity. Proteins which may be utilized according to the invention include any protein which is safe for administration to mammals and which may serve as an immunologically effective carrier protein. In particular embodiments, cell surface proteins, membrane proteins, toxins and toxoids may be used. Criteria for safety would include absence of primary toxicity and minimal risk of allergic reaction. Diphtheria and tetanus toxoids fulfill these criteria; that is, suitably prepared they are non-toxic, and the incidence of allergic reactions is acceptably low. Although the risk of allergic reaction may be significant for adults, it is minimal for infants.

According to additional particular embodiments of the invention, appropriate carrier proteins include, but are not limited to *Salmonella* flagellin, *Haemophilus* pilin, *Pseudomonas* pili, *Pseudomonas* exotoxin, outer membrane proteins of *Haemophilus* (15 kDa, 28-30 kDa, and 40 kDa membrane proteins) or *N. meningitidis* or *N. gonorrheae*, *Escherichia coli* heat labile enterotoxin LTB, cholera toxin, pneumolysin of *S. pneumoniae*, and viral proteins including rotavirus VP7 and respiratory syncytial virus F and G proteins. Additionally, there

are many carrier proteins known in the art including, but not limited to, keyhole limpet hemocyanin, bovine serum albumin, and diphtheria toxin cross-reactive mutant protein ("CRM"). Additionally, there are several methods known in the art for conjugating endotoxin to a carrier protein. Such methods may include, but are not limited to, the use of glutaraldehyde, or succinimidyl *m*-maleimidobenzoate, or 1-ethyl-3-(3-dimethyl-aminopropyl)carbodiimide, or by using bromoacetylated carrier protein (see, e.g. Robey et al., 1989, *Anal. Biochem.* 177:373-377). Conjugation of *htrB* endotoxin to a carrier protein toxin may reduce toxicity of the carrier protein toxin, but residual toxicity may remain. Further detoxification may be accomplished by means known in the art such as employing formalin which reacts with free amino groups of the carrier protein toxin.

Alternatively, native carrier protein toxin may be detoxified with formalin to produce a conventional toxoid before conjugation to the *htrB* mutant endotoxin. However, the prior formalin treatment reduces the number of free amino groups available for reaction during the conjugation process. CRM have an advantage in that they have no inherent toxicity yet none of their amino groups are occupied by the formalin. In the case of CRM197, which is immunologically identical to native toxin, treatment with formalin (though there is no need to detoxify) greatly enhances the immunological response. It is thought that this is due to stabilization of the molecule against degradation by mechanisms of the body and/or aggregation by cross-linking (immunogenicity of particles increases with size). While tetanus and diphtheria toxins are desirable carrier proteins, there may be other candidate carrier proteins which may also be suitable. Such other candidates for carriers include toxins and proteins of pseudomonas, staphylococcus, streptococcus, pertussis, and *E. coli*.

Conjugation of endotoxin to carrier proteins can be performed by a variety of methods such as by direct conjugation to the carrier protein by cyanogen bromide, reductive amination, or by using bifunctional linkers. Such bifunctional linkers include, but are not limited to, N-hydroxy succinimide-based linkers cystamine, glutaraldehyde, and diamino hexane. *htrB* endotoxin is modified to contain sulfhydryl groups withthe use of N-hydroxy succinimide-based linkers, or by the use of carbodiimide-mediated condensation of cystamine. The sulfhydryl-containing *htrB* endotoxin intermediates are then reacted to a carrier protein that has been derivatized with N hydroxy succinimidyl bromo acetate. Preferably, sulfhydryl groups are exposed on the conjugating *htrB* endotoxin for making a thiol linkage with the bromoacetylated carrier protein.

In a specific embodiment of the invention, *htrB* mutant endotoxin may be conjugated to a carrier protein, such as CRM, by using long chain sulfo N-succnimidyl 3-(2-pyridylthio)-propionate to thiolate the primary amino group(s) of the endotoxin. Long chain sulfo N-succnimidyl 3-(2-pyridylthio)-propionate was added to approximately 13 mg of the saccharide component of the *htrB* endotoxin in 0.1 M $NaHCO_3$ pH7.0 at a ratio of 1:1 (w/w) and incubated for an hour at room temperature. The mixture is then purified by gel filtration. The long chain sulfo N-succnimidyl 3-(2-pyridylthio)-propionate derivatized fractions were pooled. The N-pyridyl disulfides present in the derivatized fractions were reduced with 100 mM dithiothreitol, and purified by gel filtration. The thiolated endotoxin fractions were then collected. CRM197 was bromoacetylated by adding bromoacetic acid N hydroxy succinimide in a small volume of dimethyl formamide dropwise to CRM (in 0.1 M $NaHCO_3$) at a ratio of 1:1 (w/w) at 4°C. The solution was mixed and incubated for 1 hour at room temperature. The

reaction mixture was then purified by gel filtration, and the fractions containing bromoacetylated protein were collected. Derivatization of amino groups on carrier protein to bromoacetyl groups was monitored by a decrease in the amount of free amino groups. Bromoacetyl CRM in 0.1 M $NaHCO_3$ was added to the thiolated *htrB* mutant endotoxin at a 1:1.5 ratio of protein to endotoxin (w/w) in 0.1 M $NaHCO_3$/1 mM EDTA, and the reaction was incubated overnight at 4°C. The final conjugate was then be purified by gel filtration in a phosphate buffered saline pH 6.9.

The methods and compositions of the present invention relate to LPS and LOS biosynthetic pathways of gram-negative bacterial pathogens. More specifically, the present invention relates to mutations in the *htrB* gene of gram-negative bacterial pathogens resulting in mutant bacteria bearing endotoxin which is substantially reduced in toxicity, and yet retains antigenicity, as compared to wild type bacteria of the same species.

The genetics of lipid A biosynthesis of enteric bacteria, as it was known at the time of the present invention, is summarized in Schnaitman and Klena (1993, *Microbiol. Rev.* 57:655-682). Genes *lpxA*, *lpxB*, *lpxC*, and *lpxD* encode gene products which function on the glucosamine backbone of lipid A including transfer of β-hydroxymyristic acid to glucosamine. The *htrB* gene was described as a gene that affects the inner core structure (KDO, heptose, phosphorylethanolamine (PEA)) which was discovered during a screen for genes necessary for growth of *Escherichia coli* at elevated temperatures. Knockout mutations of *htrB* resulted in mutant *E. coli* which exhibited a reduced sensitivity to deoxycholate, an inability to grow at temperatures above 32.5°C, and a decrease in LPS staining intensity (Schnaitman et al., 1993, *supra*; Karow et al., 1992, *J. Bacteriol.* 174:7407-

7418). Karow et al. further noted that at between about 30°C to about 42°C, *E.coli htrB* mutants have changes in the fatty acid composition of both LPS and phospholipids, and particularly, overproduce phospholipids, as compared to wild type. However, it was neither known nor suggested which one or more of the at least six molecules of ester- or amide bound fatty acids is lacking in the lipid A portion of LPS of *htrB* mutants. Also no mention was made that *htrB* mutants contained a lipid A moiety specifically lacking one or both non-hydroxylated (secondary acyl chain) fatty acids responsible for endotoxicity; i.e. that the *htrB* mutant contained an altered endotoxin exhibiting reduced *in vivo* toxicity, but retaining antigenicity ("*htrB* endotoxin"), as compared to wild type.

The discoveries comprising the present invention include the unexpected results that knockout mutations of the *htrB* gene of gram-negative bacteria (including the family *Enterobacteriaceae*) result in *htrB* mutants which specifically lack one or more secondary acyl chain fatty acids which are ester-bound to the hydroxyl groups of two of the four molecules of β-OH (as shown in FIG. 2). Thus, it appears that the HtrB protein has either acyltransferase activity, or indirectly or directly affects regulation of acyltransferase activity. The following examples are presented to illustrate preferred embodiments of aspects of the present invention, and are not intended to limit the scope of the invention. In particular, a preferred embodiment is the making of an *H. influenzae htrB* mutant, and methods of using the same as a whole cell, or to isolate therefrom the endotoxin, in vaccine preparations or to generate antibodies for therapeutic or diagnostic applications. However, since the lipid A moiety is highly conserved among bacteria of the family *Enterobacteriaceae* and closely related gram-negative

bacteria, the invention relates to gram-negative bacterial pathogens, as defined previously herein. There is microheterogeneity in terms of the length of the secondary acyl chain (12 or 14 carbon chains) and to which of the four β-OH are substituted (1, 2, or 4) (Erwin et al., 1991, *Infect Immun* 59:1881-1887); however, the nature of the substitution is the same and thus the particular steps (genes and gene products) involved in the biosynthetic pathway appear conserved. For example, removal of secondary acyl chains from various gram-negative bacterial pathogens (*E. coli, H. influenzae, P. aeruginosa, S. typhimurium,* and *N. meningitidis*) using human acylxyacyl hydrolase resulted in deacylated LPS from all species tested having significantly reduced mitogenic activity (Erwin et al., 1991, *supra*) as compared to the respective wild type strain.

EXAMPLE 1

Identification of an *htrB* gene, and generation of *htrB* mutants

By complementing a nontypable *H. influenzae* strain 2019 with a *S. typhimurium rfaE* mutant strain, the *rfaE* gene of *H. influenzae* strain 2019 was cloned (Lee et al., 1995, *Infect Immun* 63:818-824). Sequence analysis of the upstream region of the *H. influenzae rfaE* gene revealed an open reading frame highly homologous to the *E. coli htrB* gene. The *H. influenzae htrB* gene comprises 933 bases and encodes a protein, HtrB, of 311 amino acids (SEQ ID NO:1) and an estimated molecular size of 36 kilodaltons (kDa). Comparison of the deduced amino acid sequence of the *H. influenzae* HtrB with the *E. coli* HtrB revealed shared homology (56% identity and 73% similarity). Cloning the *htrB* gene of *H. influenzae* into a plasmid, and subsequent *in vitro*

transcription-translation analysis, revealed that HtrB has an apparent molecular size of 32-33 kDa.

There are various standard techniques known to those skilled in the art for mutating a bacterial gene. Those techniques include site-directed mutagenesis, and shuttle mutagenesis using transposons. In one aspect of this embodiment, mutagenesis of the *htrB* gene was carried out by shuttle mutagenesis. A derivative of the bacterial transposon Tn3, mini-Tn3 (Seifert et al., 1986, *Proc. Natl. Acad. Sci. USA* 83:735-739), was used as an insertion sequence to mutate the *htrB* gene. A 2.4 kilobase (kb) *Bgl*II containing the *htrB* gene from *H. influenzae* was cloned into a plasmid which was used as a target for mini-Tn3 transposon mutagenesis. Briefly, introduced into a single bacterial cell (*E. coli*), is the plasmid containing the *htrB* gene; a plasmid immune to Tn3 transposition and containing transposase (which mediates the cointegration between Tn3 and the target molecules); and a plasmid containing mini-Tn3.

After allowing for transposition, the bacterial cells are mated with an *E. coli* strain containing the cre enzyme that is used to resolve cointegrates in shuttle mutagenesis. Transconjugates were selected for with antibiotics (kanamycin, ampicillin, and streptomycin) and analyzed by restriction endonuclease digestion.

Two plasmids, termed pB28 and pB29, each with a mini-Tn3 transposon containing the chloramphenicol acetyltransferase (CAT) gene inserted into the *htrB* open reading frame at a different location. Each plasmid was used to transform nontypable *H. influenzae* strain 2019 and bacterial cell transformants were selected for by growth in the presence of chloramphenicol (1.5 μg/ml), resulting in identification of mutant strains designated NTHi B28 and B29, respectively. Locations of the mTn3 insertion in the chromosomes of the NTHi mutants were

confirmed by genomic Southern hybridization using the 2.4 kb *Bgl*II fragment as a probe. In particular, a *Bgl*II digest of NTHi strain 2019 DNA resulted in a 2.4 kb fragment; whereas similar digests of DNA from mutants NTHi B28 and B29 revealed 4.0 kb fragments. Further, the 4.0 kb fragments were digested by *Eco*RI which is present in the mTn3.

Alternatively, methods are known in the art to perform site-directed mutagenesis into a bacterial gene (See for example, Halladay, 1993, *J. Bacteriol.* 175:684-692), and recombination of the mutated bacterial gene into the bacterial chromosome. A selectable kanamycin resistance cassette may be used to insert into, and mutate, the *htrB* gene contained within a shuttle plasmid. Subsequent transformation into a bacterial host cell with the shuttle plasmid, and recombination of the bacterial genome (at the site of the genomic copy of the *htrB* gene) with the cassette via *htrB* flanking sequences, results in the site-directed mutagenesis of the bacterial *htrB* gene.

Primer extension analysis can be used to determine the promoter region of the *htrB* gene. The *H. influenzae htrB*'s promoter region was determined by primer extension analysis by first growing the bacteria, harvesting and purifying the RNA, and using a commercial primer extension kit according to the manufacturer's suggestions. A single transcription site was found using a primer (SEQ ID NO:2) complementary to the 5' region of the *htrB* open reading frame. The first nucleotide was a cytosine (C) residue located at 21 bp upstream of the putative translation start site, ATG. The region upstream of the transcription start site contained a sequence (SEQ ID NO:1, bases 13 to 29) similar to the consensus -10 region of the bacterial σ^{70}-dependent promoters at an appropriate distance. An

element (SEQ ID NO:1, bases 1 to 6) resembles the consensus sequence of the -35 region.

EXAMPLE 2

Characterization of *htrB* mutants

Growth Characteristics

NTHi B28 and B29 strains were initially selected at 30°C, and were unable to grow at 37°C. With further passages at 30°C, the NTHi *htrB* mutants began to lose temperature sensitivity and demonstrated a slow rate of growth, as compared to NTHi 2019, at 37°C. However, for growth temperatures greater than or equal to 38.5°C, the temperature sensitivity remained for the *htrB* mutants.

It was reported previously that *E. coli htrB* mutants demonstrated a change in membrane permeability to various compounds including kanamycin and deoxycholate (Karow et al., 1992, *supra*). The NTHi *htrB* mutants were also tested for sensitivity to kanamycin and deoxycholate. Overnight cultures grown at 30°C were then diluted and allowed to grow in the presence of 5 μg/ml kanamycin at either 30°C or 37°C. At 30°C, no difference was detected in the growth rate between NTHi 2019 and the NTHi *htrB* mutant strains in the absence of kanamycin. However, the growth of the *htrB* mutants was significantly inhibited in the presence of kanamycin, whereas NTHi 2019 was not affected. For the *htrB* mutants , the sensitivity to kanamycin was even greater at 37°C, since the mutants failed to show growth in the presence of kanamycin at 37°C. Likewise, at 30°C the *htrB* mutants showed sensitivity, as compared to NTHi strain 2019, at concentrations of greater than 500 μg/ml deoxycholate, and failed to grow at 1000 μg/ml. At 37°C, the *htrB* mutants showed almost complete inhibition of growth in the presence of only 250 μg/ml deoxycholate.

Endotoxin Characteristics

The LPS of *E. coli htrB* mutants has been characterized as being weakly stained on silver-stained polyacrylamide gels, but its migration pattern did not vary as compared to LPS from wild type. In contrast, the LOS from NTHi mutants B28 and B29 migrated faster than that from NTHi strain 2019 on silver-stained gels. Additionally, the LOS from the B28 and B29 mutants displayed a brownish color rather than black, as displayed by NTHi 2019. Reconstitution, by introducing a plasmid with an intact *htrB* gene into the mutant, of NTHi mutant B29 confirmed that the differences in growth characteristics and LOS migration and staining were due to mutation of the *htrB* gene.

The NTHi *htrB* mutant LOS and wild type LOS were each analyzed by electrospray ionization-mass spectrometry (ESI-MS) to provide molecular mass profiles for the different components of LOS. First, LOS was isolated from the respective strains. LPS or LOS can be isolated by the phenol-water method (Westphal et al., 1965, *Methods in Carbohydrate Chemistry* 5:83-91); or using an alternative purification procedure (using a protease; Hitchcock et al., 1983, *J. Bacteriol.* 154:269-277). The isolated LOS species were then O-deacylated by mild hydrazine treatment (37°C for 20 minutes; see Phillips et al., 1990, *Biomed. Environ. Mass Spectrom.* 19:731-745). Analysis by ESI-MS of the different LOS species showed that while the O-deacylated LOS from NtHi mutant B29 and NTHi 2019 were similar in molecular mass profile, two differences can be clearly discerned. In the *htrB* mutant, there is a decrease (50% reduction) in the amount of LOS containing two phosphoethanolamines (PEA) in the inner core structure; and there is a shift to high molecular weight LOS species containing more hexoses. These findings suggest that the degree of phosphorylation may be affecting chain progression from

specific heptose moieties, and that HtrB either directly or indirectly affects phosphorylation of LOS.

Mass spectrometry was used to analyze the lipid A. More specifically, lipid A from *htrB* mutant LOS and from wild type LOS were each analyzed by liquid secondary ion mass spectrometry (LSIMS) in the negative ion mode to provide a spectrum of molecular ions for the different components lipid A. First, the LOS species were each hydrolyzed in 1% acetic acid for 2 hours at 100°C at a concentration of 2 mg/ml. The hydrolysates were centrifuged, and the supernatants removed. The water soluble crude lipid A fractions were washed twice in water, and once in an organic mixture (chloroform/methanol/water; by volume 2:1:1) and then evaporated to dryness. For analysis, the lipid samples were redissolved in CH_2Cl_2/CH_3OH (3:1, v/v) and 1 μl of nitro-benzylalcohol/triethanolamine (1:1, v/v) and applied as a liquid matrix onto a mass spectrometer. LSIMS of the wild type (NTHi 2019) revealed a spectrum containing two deprotonated molecular ions consistent with a hexaacyl lipid A structure containing either one (hexaacyl monophosphoryl lipid A, M_r = 1744) or two phosphates (hexaacyl diphosphoryl lipid A, M_r = 1824). This spectrum is essentially identical to that reported for the lipid A structure of LOS of *H. ducreyi* (Melaugh et al., 1992, *J. Biol. Chem.* 267:13434-13439). The lower mass fragments are believed to be ions which arise through LSIMS-induced fragmentation of higher ass mono- and diphosphorylated molecular ion species.

In contrast, the LSIMS spectrum for the lipid A preparation from the *htrB* mutant LOS lacks molecular ions corresponding to the wild type hexaacyl lipid A species. There are two high mass ions which correspond to the molecular ions for a mono- and diphosphoryl pentaacyl lipid A species missing one of the secondary acyl chains (e.g. myrisitic acid moiety). Further, at

the lower masses are two additional molecular ion species that correspond to a mono- and diphosphoryl tetraacyl lipid A species lacking both secondary acyl chains. In summary, the lipid A structure of the wild type's LOS is hexaacyl; whereas the lipid A structure of the *htrB* mutant shows two species, a tetraacyl (Fig. 2A) and a pentaacyl species (FIG. 2B) indicating the loss of at least one, and sometimes both secondary acyl chains. The *htrB* mutant is comprised of approximately 90% tetraacyl lipid A with only the four hydroxymyristic acid ester and amide-linked fatty acids, and approximately 10% pentaacyl lipid A with one myristic acid substitution.

EXAMPLE 3

Substantially reduced toxicity of *htrB* mutants

The effect due to the lack of one or more secondary acyl chains on the toxicity of a gram-negative bacterial pathogen was examined using a standard *in vitro* assay for measuring *in vivo* toxicity. Murine macrophage-like cell line J774, when stimulated by endotoxin, secretes TNFα. The amount of TNFα, a directly proportional to the toxicity of the stimulating LPS or LOS, can be measured by (a) removing the cell-free supernatant containing the TNFα; (b) adding the supernatant to a TNFα-sensitive cell line, such as WEHI 164; and (c) measuring the resultant cytotoxicity (See for example, Espevik et al., 1986, *J Immunol Methods* 95:99; Sakurai et al., 1985, *Cancer Immunol Immunother* 20:6-10; Adams et al., 1990, *J Clin Microbiol* 28:998-1001; Adams et al., 1990, *J Leukoc Biol* 48:549-56; Tsai et al., 1992, *Cell Immunol* 144:203-16; and Pfister et al., 1992, *Immunol* 77:473-6).

In this assay, adherent J774 cells were removed from culture, washed with PBS-1mM EDTA, and then washed twice with complete tissue culture medium without

antibiotics. $2x10^6$ to $4x10^6$ J774 cells/100 mm culture dish were incubated in tissue culture medium overnight in a CO_2 incubator. Adherent J774 cells are removed with PBS-1mM EDTA, washed three times in tissue culture medium, and adjusted to $5x10^5$/ml. Aliquots of 50μl were added per well of a round bottom 96 well plate. The plate is then incubated for 1 hour at 37°C in a CO_2 incubator. Per well is added either an *htrB* mutant, or the wild type strain, in various colony forming units (cfu, infection dose). The plate is then incubated at 37°C for 1 hour in a CO_2 incubator. After the incubation, 100μl of culture medium containing 50μg/ml gentamycin is added per well. The plate is then incubated overnight at 37°C in a CO_2 incubator. Aliquots of 50μl of the J774 supernatant were removed per well and transferred into wells of a flat bottom 96 well plate. Serial 10 fold dilutions were made of the J774 supernatant. Included as a control is a dilution series of recombinant TNFα (rTNFα). Added per well is 50μl of WEHI 164 clone 13 cells at $6x10^5$ cells/ml in tissue culture medium + 25 mM LiCl + 2μg/ml actinomycin D; and the mixture was incubated overnight at 37°C in a CO_2 incubator. After the incubation, 10μl of alomar blue is added, and 5-7 hours later the optical density is read at 570 nm. The assay utilizes alomar blue as a color indicator; i.e., alomar blue is converted to a red color by living cells, but remains blue if the cells are killed.

FIG. 3 shows a comparison between the number of bacterial cells of *H. influenzae* strain 2019 (wild type, □), and of bacterial cells of *htrB* mutant NTHi B29 (O and Δ) necessary to stimulate the release of enough TNFα from J774 cells to kill the TNFα-susceptible cell line WEHI 164. $B29_{hi}$ (Δ) and $B29_{LO}$ (O) refer to a high number (>3) and low number (<3) of passages of *htrB*

mutant, respectively. As shown in FIG. 3, the *htrB* mutant shows a reduced ability to stimulate TNFα release; i.e., between an approximately 10 fold reduction ($B29_{LO}$) to an approximately 100 fold reduction ($B29_{hi}$). This reduced ability to stimulate TNFα is one indication of the *htrB* mutant being substantially reduced in toxicity due to the lack of one or more secondary acyl chains in the lipid A portion of the endotoxin.

The effect due to the lack of one or more secondary acyl chains on the toxicity of a gram-negative bacterial pathogen was also examined using a standard *in situ* assay for measuring *in vivo* toxicity. SV-40 transformed human respiratory epithelial cells and human primary respiratory epithelial cells, when stimulated by endotoxin, produces TNFα which production can be demonstrated by detection of TNFα mRNA using methods known to those skilled in the art for *in situ* hybridization (a modification of MacNaul et al., 1990, *J. Immunol.* 145:4154-66). The cells are grown in a monolayer within wells of a 24-well plate until approximately confluent. To stimulate the cells, 1μg/ml of LOS is added, and the cells are incubated overnight at 37°C. A counterstain (e.g., membrane stain) is added and the cells are then fixed with 0.5 ml of 2% paraformaldehyde in buffer so that the counterstain is fixed directly into the cells. Hybridization is then performed using an oligonucleotide probe specific for TNFα RNA (SEQ ID NO:4), and a control probe (SEQ ID NO:5). The amount of TNFα mRNA visually detected is directly proportional to the toxicity of the stimulating LPS. TNFα mRNA was minimally induced in SV-40 transformed human respiratory epithelial cells, and human primary respiratory epithelial cells (FIG. 4), exposed to LOS isolated from the *htrB* mutant NTHi B29. This is in contrast to LOS isolated from parent strain NTHi 2019 which induced high

levels of TNFα mRNA in these human respiratory cells (FIG. 4) and cell lines.

The substantial reduction in toxicity exhibited by the *htrB* mutant, as observed by the TNFα assays, due to the lack of one or more secondary acyl chains is further supported by previously reported assays of bioactivity of endotoxin treated with acyloxyacyl hydrolase which selectively removes the secondary acyl chains from endotoxin. Deacylated endotoxin from *E. coli*, *H. influenzae*, *N. meningitidis*, and *S. typhimurium* were (a) similarly reduced in potency in the Limulus lysate test relative to the respective wild type endotoxin; (b) reduced in the ability to stimulate neutrophil adherence to human endothelial cells relative to the respective wild type endotoxin; and (c) reduced in mitogenic activity for murine splenocytes (Erwin et al., 1991, *Infect Immun* 59:1881-1887); yet maintained expression of antigenic epitopes. Similarly, *S. typhimurium* LPS treated with acyloxacyl hydrolase showed a reduction in toxicity by 100-fold or greater in a dermal Shwartzman reaction; was less pyrogenic in a thermal response model; showed a 5 to 12 fold reduction in B-cell mitogenicity; and showed a 10 to 20 fold reduction in the release of prostaglandin E_2, as compared to wild type endotoxin, in concluding that maximally deacylated LPS was at least 10 fold less toxic than wild type endotoxin (U.S. Patent No. 4,929,604).

EXAMPLE 4

Use of *htrB* mutants as immunogens

In one aspect of this embodiment, the *htrB* mutant of a gram-negative bacterial pathogen is used as a whole cell vaccine. The benefit of using live, attenuated (weakened in its ability to cause pathogenesis) bacteria as an immunogen in a vaccine formula is that they are able to survive and may persist

in the human or animal body, and thus confer prolonged immunity against disease. In conjunction with the benefit of using a live bacteria to prolong the immune response, gram-negative bacterial pathogen *htrB* mutants have the added benefit in that they exhibit substantially reduced toxicity. Another advantage, as compared to a vaccine formulation comprising an isolated peptide representing a bacterial antigen, is that a bacterial antigen expressed on the surface of a bacterial cell will often result in greater stimulation of the immune response. This is because the surface of bacteria of the family *Enterobacteriaceae* acts as a natural adjuvant to enhance the immune response to an antigen presented thereon (Wezler, 1994, *Ann NY Acad Sci* 730:367-370). Thus, using a live bacterial vaccine, such as an *htrB* mutant, to express complete proteins in an native conformation (i.e., as part of the bacterial outer membrane) is likely to elicit more of a protective immune response than an isolated protein alone.

Live bacterial vaccine vectors of the family *Enterobacteriaceae* that have been described previously include attenuated *Salmonella* strains (Stocker et al., U.S. Patent Nos. 5,210,035; 4,837,151; and 4,735,801; and Curtiss et al., 1988, Vaccine 6:155-160; herein incorporated by reference), and *Shigella flexneri* (Sizemore et al., 1995, *Science* 270:299-302; herein incorporated by reference). One preferred embodiment is to provide a vaccine delivery system for human or animal (depending on the genus and species of the gram-negative bacterial pathogen) mucosal pathogens. Thus, immunization by the parental route or by the mucosal route with a prophylactically effective amount of the *htrB* mutant, or an *htrB* mutant transformed to recombinantly express additional bacterial antigens (that do not negatively affect the growth or replication of the transformed *htrB* mutant), can lead to

colonization of mucosal surfaces to induce mucosal immunity against the antigens displayed on the surface of, or secreted from the *htrB* mutant. The resultant *htrB* mutant can be used in a vaccine formulation which expresses the bacterial antigen(s).

Similar methods can be used to construct an inactivated *htrB* mutant vaccine formulation except that the *htrB* mutant is inactivated, such as by chemical means known in the art, prior to use as an immunogen and without substantially affecting the immunogenicity of the expressed immunogen(s). For example, human bronchial mucosal immunity has been stimulated with an aerosol vaccine comprising lysed *H. influenzae* (Latil et al., 1986, *J Clin Microbiol* 23:1015-1021). Either of the live *htrB* mutant vaccine or the inactivated *htrB* mutant vaccine may also be formulated with a suitable adjuvant in order to further enhance the immunological response to the antigen(s) expressed by the vaccine vector, as to be described in more detail.

In another aspect of this embodiment, the endotoxin is isolated from the *htrB* mutant using methods known in the art, and the isolated *htrB* endotoxin is used in a vaccine formulation. As mentioned previously, major antigenic determinants of gram-negative bacteria are believed to reside in the carbohydrate structure of the O-specific side chain of LPS and the complex carbohydrate structure of LOS. However, the chemical nature of LPS and LOS prevent the use of these molecules in vaccine formulations; i.e., active immunization with LPS or LOS is unacceptable due to the inherent toxicity of the secondary acyl chains of the lipid A portion of endotoxin. The endotoxin isolated from an *htrB* mutant of a gram-negative bacterial pathogen lacks one or more secondary acyl chains, and thus exhibits substantially reduced toxicity as compared to endotoxin isolated from the respective wild type bacteria. Therefore, endotoxin

isolated from an *htrB* mutant of a gram-negative bacterial pathogen can be used in a vaccine formulation in inducing immunity against the respective wild type gram-negative bacterial pathogen. LPS or LOS can be isolated by the phenol-water method (Westphal et al., 1965, *Methods in Carbohydrate Chemistry* 5:83-91); or using an alternative purification procedure (using a protease; Hitchcock et al., 1983, *J. Bacteriol.* 154:269-277).

Many methods are known for the introduction of a vaccine formulation into the human or animal (collectively referred to as "individual") to be vaccinated. These include, but are not limited to, intradermal, intramuscular, intraperitoneal, intravenous, subcutaneous, ocular, intranasal, and oral administration. Conventionally, vaccine formulations containing either live bacteria, or attenuated or inactivated bacteria, are administered by injection or by oral administration. For example, respiratory immunity can be stimulated by intestinal immunization with purified *H. influenzae* antigens (Cripps et al., 1992, *J. Infect Dis* 165S1:S199-201; herein incorporated by reference). The vaccine formulation may comprise a physiologically acceptable solution as a carrier in which the *htrB* mutant bacterial cells or isolated *htrB* mutant endotoxin is suspended. Various adjuvants may be used in conjunction with vaccine formulations. The adjuvants aid by modulating the immune response and in attaining a more durable and higher level of immunity using smaller amounts of vaccine antigen or fewer doses than if the vaccine antigen were administered alone. Examples of adjuvants include incomplete Freund's adjuvant, Adjuvant 65 (containing peanut oil, mannide monooleate and aluminum monostearate), oil emulsions, Ribi adjuvant, the pluronic polyols, polyamines, Avridine, Quil A, saponin, MPL, QS-21, and mineral gels

such as aluminum hydroxide, aluminum phosphate, etc. The vaccine formulation is administered in a prophylactically effective amount to be immunogenic, which depends on factors including the individual's ability to mount an immune response, the degree of protection to be induced, and the route of administration.

In another aspect of the invention, the vaccine formulation can be administered orally by including it as part of the feed given to economically important livestock. As known by those skilled in the art, species of *Haemophilus, Campylobacter, Pseudomonas,* and *Salmonella* are pathogenic for economically important livestock. Using the methods according to the present invention, as illustrated in the following examples, *htrB* mutants of such animal pathogens can be produced. The resultant *htrB* mutants, or endotoxin isolated therefrom, can be used in a vaccine formulation. Use of vaccine formulations, containing one or more antigens of various microbial pathogens, in animal feed has been described previously (See for example, Pritchard et al., 1978, *Avian Dis* 22:562-575).

EXAMPLE 5

H. influenzae htrB mutants as immunogens

In one embodiment, the *htrB* mutant is an *H. influenzae htrB* mutant. *Haemophilus influenzae* is an important human respiratory tract pathogen in diseases including otitis media, chronic sinusitis, and chronic obstructive pulmonary disease. Certain surface-exposed bacterial components, including P2, P6, and LOS, appear to be antigens which may confer a protective immune response in immunized humans. Such antigens have been shown to be targets of bactericidal antibody, and the presence of serum bactericidal antibody is associated

with protection from infection by *H. influenzae* (Faden et al., 1989, *J. Infect. Dis.* 160:999-1004).

5.1 In one aspect of the this embodiment, the endotoxin isolated from an *htrB* mutant is used as the immunogen in a vaccine formulation. As demonstrated in Example 3 herein, the endotoxin isolated from an *htrB* mutant of a gram-negative bacterial pathogen lacks one or more secondary acyl chains, and thus exhibits substantially reduced toxicity as compared to endotoxin isolated from the respective wild type bacterial pathogen. Therefore, endotoxin isolated from an *htrB* mutant of *H. influenzae* can be used in a vaccine formulation in inducing immunity against the respective wild type strain. The *htrB* mutant LOS may be isolated by a method known to those skilled in the art for isolating LOS. The *htrB* mutant LOS may be used in a vaccine formulation containing one or more agents selected from the group consisting of a pharmaceutically acceptable carrier (e.g., a physiological solution), an adjuvant, or a carrier protein.

To illustrate the effects of immunization with *htrB* mutant LOS, a mouse model was used. NTHi 2019 LOS, and *htrB* mutant NTHi B29 LOS were each isolated from their respective strains using the phenol-water method. Groups of at least 5 Swiss Webster mice were immunized subcutaneously with 1 μg of either NTHi 2019, *htrB* mutant B29 LOS, or *htrB* mutant conjugated to a carrier protein, with adjuvant QS-21. Sera was collected from each group 5 weeks after immunization, and the sera from animals comprising a group were pooled. The pooled sera was assessed for titer of anti-LOS antibody by enzyme-linked immunosorbent assay (ELISA). Microtiter wells of ELISA plates were coated with either 10 μg of NTHi 2019, or *htrB* mutant B29 LOS. FIG. 5 illustrates the mean titers of anti-LOS antibody against NTHI 2019 LOS (antigen coating) in ELISA from mice immunized with NTHi

2019 (Pool 595), *htrB* mutant B29 LOS (Pool 597), or *htrB* mutant B29 LOS conjugated to a carrier protein (Pool 606). FIG. 6 illustrates the mean titers of anti-LOS antibody against *htrB* mutant B29 LOS (antigen coating) in ELISA from mice immunized with NTHi 2019 (Pool 595), *htrB* mutant B29 LOS (Pool 597), or *htrB* mutant B29 LOS conjugated to a carrier protein (Pool 606). Pre-immune sera was also included as a control ("week 0" or "time 0").

The antibody induced by the different LOS preparations were then tested for functional activity by performing bactericidal assays using NTHi 2019 as the target. Into the wells of a 96-well plate are added 0.150 ml buffer, 0.040 ml pooled human sera as a complement source, and 0.01ml of NTHi 2019. Typically, the organism is plated in a dilution (e.g. 20 to 200 cfu). Into test wells are added the respective antisera either undiluted ("neat"), 1/10 dilution, or 1/100 dilution. The plate is then rotated vigorously (175-200 rpm) at 37°C for 30 minutes. Aliquots from respective wells are plated on media and grown to determine percentage survival, and log kill. Log kill is calculated as the log(cfu 30 minutes/cfu time 0). The results of the bactericidal assay are shown in Table 1, where Group R595 is the antisera induced by NTHI 2019 LOS, Group R597 is the antisera induced by *htrB* mutant B29 LOS, and Group R606 is the antisera induced by *htrB* mutant B29 LOS conjugated to a carrier protein.

Table 1

Group	week	dilution	% survival	log kill
R595	0	neat	1.00	-1.99
		1/10	37.30	-0.43
		1/100	87.20	-0.06
	5	neat	-	-4.49
		1/10	0.07	-3.15

		1/100	20.20	-0.70
	7	1/10	-	-4.62
		1/100	7.80	-1.11
R597	0	neat	3.50	-1.46
		1/10	62.40	-0.20
		1/100	108.60	0.04
	5	neat	-	-4.49
		1/10	4.40	-1.36
		1/100	82.40	-0.08
	7	1/10	-	-4.49
		1/100	99.80	0.00
R606	0	neat	0.13/110.5	-2.87/0.92
		1/10	130.20	0.11
		1/100	106.50	0.03
	5	neat	1.5/0.1	-1.81/-2.9
		1/10	25.10	-0.60
		1/100	164.10	0.22
	7	1/10	0.04	-3.44
		1/100	185.90	0.27

The greater the log kill (the more negative the number, e.g. -4.49), the greater the bactericidal activity is of the respective antibody. Thus, for comparison purposes, at week 7 and at a 1/10 dilution, log kill for the antisera induced by NTHi 2019 LOS is -4.62, the log kill for the antisera induced by NTHi *htrB* mutant B29 LOS is -4.49, and the log kill of the antisera induced by *htrB* mutant B29 LOS conjugated to a carrier protein is -3.44. By ELISA, it looks like the antisera induced by *htrB* mutant B29 LOS antibody is low in titer; yet, as demonstrated by the bactericidal assays, significant functional antibody is raised by immunization with *htrB* mutant B29 LOS.

5.2 In another aspect of the this embodiment, *htrB* mutant bacterial cells are used as the immunogen in a vaccine formulation. To illustrate the effects of immunization with *htrB* mutant bacterial cells, an infant rat model was used. The use of the infant rat model as a model of bacteremic infections due to type b *H. influenzae* (Hib) in humans, and for determining the virulence of type b *H. influenzae* strains, has been accepted by those skilled in the art (see, e.g., Smith et al., 1973, *Infect. Immun.* 8:278-290; Moxon et al., 1974, *J. Infect. Dis.* 129:154-62; Rubin et al., 1983, *Infect. Immun.* 41:280-284; Zwahlen et al., 1985, *J. Infect. Dis.* 152:485-492).

Type b strain A2 of *H. influenzae* has already been characterized as a highly virulent strain in the infant rat model system that causes bacteremia and meningitis after inoculation (e.g., intraperitoneal or intranasal), and as a clinical isolate from humans (it isolated from a child with meningitis due to *H. influenzae*). Using the methods according to Example 1, an *htrB* mutant was made from Hib strain A2. One week old infant Sprague-Dawley albino rats were inoculated intraperitoneally with either 10^7 Hib strain A2 or 10^7 *htrB* A2 mutant and then assessed for intravascular clearance by measuring the number of colony forming units (cfus) per ml of blood obtained 48 hours post-inoculation. The results showed that 20 of 20 infant rats inoculated with Hib strain A2 showed bacteremia, with all rats showing greater than 10^5 cfu/ml of strain A2. In contrast, only 13 of 20 infant rats inoculated with *htrB* A2 mutant showed bacteremia, with only 10 of the 13 showing greater than 10^5 cfu/ml of *htrB* A2 mutant.

Similarly, one week old infant Sprague-Dawley albino rats were inoculated intranasally with either 10^7 Hib strain A2 or 10^7 *htrB* A2 mutant and then assessed for intravascular clearance. The results showed that 8 of

20 infant rats inoculated with Hib strain A2 showed bacteremia, with 7 of those 8 rats showing greater than 10^5 cfu/ml of strain A2. In contrast, none of the 30 infant rats inoculated with *htrB* A2 mutant showed bacteremia. Taken together, it can be concluded from this model system that *htrB* mutants demonstrate attenuated virulence, as compared to its wild-type strain, as indicated by the decreased ability to cause bacteremia (e.g., a 30% reduction in the occurrence of bacteremia).

To further illustrate the effects of immunization with *htrB* mutant bacterial cells, a chinchilla model was used. The use of the chinchilla model as a model of middle ear infections due to nontypable *H. influenzae* (NTHi) in humans, and for determining the virulence of NTHi strains, has been accepted by those skilled in the art (see, e.g., Bakaletz et al., 1989, *Acta Otolaryngol.* 107:235-243; Madore et al., 1990, *Pediatrics* 86:527-34; Barenkamp, 1986, *Infect. Immun.* 52:572-78; Green et al., 1994, *Methods Enzymol.* 235:59-68).

NTHi 2019 is a clinical isolate described previously (see, e.g., Murphy et al., 1986, *Infect. Immun.* 54:774-779). Each healthly adult chinchilla was inoculated, via the epitympanic bulla into the middle ear space, with various log doses of either NTHi 2019 or *htrB* mutant NTHi B29. The course of middle ear disease was then assessed by periodic otoscopic examination for tympanic membrane inflammation or middle ear infusion, and aspiration from the middle ear with subsequent culture. The results showed that when compared to NTHi 2019, it takes up to a 3 log greater dose of *htrB* mutant NTHi B29 (10^7 cfu/ear) to induce middle ear infection. It can be concluded from this model system that *htrB* mutants demonstrate attenuated virulence, as compared to

its wild-type strain, as indicated by the decreased ability to cause middle ear disease.

In another aspect of this embodiment the *H. influenzae htrB* mutant is genetically engineered to express one or more heterologous bacterial antigens. As will be discussed in more detail below, *H. influenzae* has a natural genetic transformation process involving linearized DNA binding, uptake via one or more uptake sequences (e.g. AAGTGCGGT -SEQ ID NO:3), translocation, and recombination. Thus, one mechanism to introduce a recombinant DNA molecule containing the at least one heterologous bacterial antigen to be expressed, is to transform the host *H. influenzae htrB* mutant with linearized recombinant DNA molecule containing the DNA encoding the at least one heterologous bacterial antigen ("the encoding sequence"). Alternatively, the recombinant DNA molecule containing the encoding sequence to be expressed can be inserted into a plasmid vector, and either introduced into as a linearized recombinant molecule by the natural transformation process; as circularized recombinant plasmid using electroporation of noncompetent *H. influenzae htrB* mutants; or as a circularized recombinant plasmid transformed into competent *H. influenzae htrB* mutants.

Plasmids useful for cloning of and expression from recombinant DNA molecules in *H. influenzae* are known to those skilled in the art. Such plasmids include:

pRSF0885 confers ampicillin resistance, and contains a *Pvu*II cloning site and a defective TnA sequence (Setlow et al., 1981, *J. Bacteriol.* 148:804-811), and can replicate in both *H. influenzae* and *E.coli* (Trieu et al., 1990, *Gene* 86:99-102).

pDM2 was constructed by cloning chloramphenicol resistance into pRSF0885; and **pDM5** was constructed by

cloning tetracycline resistance into pRSF0885 (McCarthy et al., 1986, *J. Bacteriol.* 168:186-191).

pVT63, pVT64, pVT65, pVT66 are improved shuttle vectors for *H. influenzae* and *E. coli* based on pDM2 (Trieu et al., 1990, *Gene* 86:99-102), and contain the pUC-derivative of the ColE1 *ori*, and the pRSF0885 rep locus. Additionally, each plasmid has drug markers with unique restriction sites for insertional inactivation of the drug marker as follows: pVT63- ApR (*Hinc*II, *Pst*I, *Sca*I), KmR (*Cla*I, *Hind*III, *Nru*I, *Sma*I, *Xho*I); pVT64- ApR (*Hinc*II, *Pst*I, *Sca*I, *Ssp*I), SpR; pVT65- ApR (*Hinc*II, *Pst*I, *Sca*I, *Pvu*I, *Ssp*I), CmR (*Bal*I, *Nco*I); pVT66- ApR (*Hinc*II, *Pst*I, *Sca*I, *Pvu*I), CmR (*Sma*I).

pACYC177, pACYC184, pSU2718, pSU2719 are improved shuttle vectors for *H. influenzae* and *E. coli* based on p15A (Chandler, 1991, *Plasmid* 25:221-224), have the p15A *ori*, and were compatible with a plasmid containing the RSF0885 origin of replication. Additionally, each plasmid has multiple cloning sites restriction sites and drug markers as follows: pACYC177- ApR, KmR (Accession No. X06402); pACYC184- CmR, TcR (Accession No. X06403); pSU2718- CmR and polycloning site from pUC18 (Accession No. M64731); and pSU2719- CmR and polycloning site from pUC19 (Accession No.M64732).

pQL1 is an improved shuttle vector for use in *H. influenzae* and *E. coli* containing both the pMB1 *ori* and P15a *ori*, KmR which is flanked by *H. influenzae* uptake sequences, a multiple cloning site containing a unique *Bam*HI and *Sma*I restriction sites, and which is particularly suited for analyzing *H. influenzae* promoter strength in *H. influenzae* (Heidecker et al., 1994, *Gene* 150:141-144).

In cloning the recombinant DNA molecule containing the encoding sequence into a plasmid vector, one skilled in the art will appreciate that the choice of restriction enzymes for digesting both the

recombinant DNA molecule and the plasmid to result in compatible ends for ligation depends on the unique restriction enzyme sites at the ends of the recombinant DNA molecule, whether occurring naturally or engineered such as during enzymatic amplification; one or more unique restriction enzyme sites within the plasmid vector; whether insertion into the plasmid vector will assist in the selection process (See, e.g., pVT66); and whether a plasmid-derived promoter is used solely, or in addition to the promoter(s) of the encoding sequences, to drive expression from the recombinant DNA molecule. Selection and screening of transformed *H. influenzae htrB* mutants may be accomplished by methods known in the art including detecting the expression of a marker gene (e.g., drug resistance marker) present in the plasmid, and immunodetection of the expressed and displayed heterologous bacterial antigen. While this aspect of the embodiment illustrates that the recombinant DNA molecule containing the encoding sequence can be inserted into a plasmid and expressed in *H. influenzae htrB* mutants, it will be appreciated by those skilled in the art that vectors other than plasmids, can be used including, but not limited to, bacteriophage vectors.

Successful expression of the at least one heterologous bacterial antigen requires that either the recombinant DNA molecule comprising the encoding sequence, or the vector itself, contain the necessary control elements for transcription and translation which is compatible with, and recognized by the particular host system used for expression. Using methods known in the art of molecular biology, including methods described above, various promoters and enhancers can be incorporated into the vector or the recombinant DNA molecule containing the encoding sequence to increase the expression of the heterologous bacterial antigen, provided that the increased expression of the

heterologous bacterial antigen(s) is compatible with (for example, non-toxic to) the *htrB* mutant. As referred to herein, the encoding sequence can contain DNA encoding more than one heterologous bacterial antigen, and may include viral and/or fungal antigen-encoding sequences, to create a multivalent antigen for use as an improved vaccine composition.

The selection of the promoter will depend on the expression system used. For example, a preferred promoter in an *H. influenzae* expression system may be the P2 or P6 promoter operatively linked to the encoding sequence. Promoters vary in strength, i.e. ability to facilitate transcription. Generally, for the purpose of expressing a cloned gene, it is desirable to use a strong promoter in order to obtain a high level of transcription of the gene and expression into gene product. For example, bacterial, phage, or plasmid promoters known in the art from which a high level of transcription has been observed in a host cell system comprising *E. coli* include the lac promoter, trp promoter, recA promoter, ribosomal RNA promoter, the P_R and P_L promoters, lacUV5, ompF, bla, lpp, and the like, may be used to provide transcription of the inserted encoding sequence.

Other control elements for efficient gene transcription or message translation include enhancers, and regulatory signals. Enhancer sequences are DNA elements that appear to increase transcriptional efficiency in a manner relatively independent of their position and orientation with respect to a nearby gene. Thus, depending on the host cell expression vector system used, an enhancer may be placed either upstream or downstream from the encoding sequence to increase transcriptional efficiency. These or other regulatory sites, such as transcription or translation initiation signals, can be used to regulate the expression of the

encoding sequence. Such regulatory elements may be inserted into the recombinant DNA molecule containing the encoding sequence, or nearby vector DNA sequences using recombinant DNA methods described herein, and known to those skilled in the art, for insertion of DNA sequences.

Accordingly, a recombinant DNA molecule containing an encoding sequence, can be ligated into an expression vector at a specific site in relation to the vector's promoter, control, and regulatory elements so that when the recombinant vector is introduced into the *htrB* mutant, the heterologous bacterial antigen can be expressed in the host cell. The recombinant vector is then introduced into the *htrB* mutant, and the transformed *htrB* mutants are selected, and screened for those cells containing the recombinant vector. Selection and screening may be accomplished by methods known in the art, and depending on the vector and expression system used.

The introduction of a recombinant DNA molecule containing the encoding sequence (including an expression vector or plasmid containing the same) into *H. influenzae htrB* mutants can be accomplished in any one of three processes: a natural genetic transformation process; transformation of competent bacterial cells; and electroporation of non-competent bacterial cells.

Natural transformation process

The natural genetic transformation process of *H. influenzae* involves linearized DNA binding, uptake via one or more uptake sequences, translocation, and recombination. Thus, one mechanism to introduce a recombinant DNA molecule containing the encoding sequence to be expressed into at least one heterologous bacterial antigen, is to transform the host *H. influenzae* with linearized recombinant DNA molecule

containing the encoding sequence; or a linearized vector having inserted into it the recombinant DNA molecule containing the encoding sequence to be expressed. In this natural process, when the linearized DNA is translocated intracellularly, one of the translocated strands of DNA is apparently degraded by exonuclease activity (Barany et al., 1983, *Proc. Natl. Acad. Sci. USA* 80:7274-7278). If the translocated strand lacks homology sufficient for recombination into the *H. influenzae* chromosome, the translocated strand becomes susceptible to further degradation (Pifer et al., 1985, *Proc. Natl. Acad. Sci. USA* 82:3731-3735). Using methods known in the art (e.g., Barany et al., 1983, *supra*; herein incorporated by reference), linearized DNA containing the encoding sequence can be introduced into *H. influenzae htrB* mutants. Since the encoding sequence can be flanked by *H. influenzae* sequences, to increase the likelihood of recombination of the encoding sequence into the *H. influenzae htrB* mutants' genome is likely to occur.

Transformation of competent bacterial cells

Another mechanism to introduce a recombinant DNA molecule containing the encoding sequence to be expressed into at least one heterologous bacterial antigen, is to transform competent host *H. influenzae htrB* mutants with a circular vector, such as a plasmid, having inserted into it the recombinant DNA molecule containing the encoding sequence to be expressed. Competence of *H. influenzae* develops best under conditions in which the bacterial cell duplication is inhibited, such as a temporary shift to anaerobic conditions, by physiological change occurring during late-log phase growth, and transfer of cells into nutrient-poor, chemically-defined medium. Such defined media for the development of competence of *H. influenzae*

has been previously described in detail (Herriott et al., 1970, *J. Bacteriol.* 101:517-524; herein incorporated by reference). It appears that only a short time after entering competent *H.influenzae*, a plasmid containing sequences homologous to the bacterial chromosome can insert its homologous sequence (such as the encoding sequence flanked by *H. influenzae* sequences) into the chromosome via recombination (Setlow et al., 1981, *supra*). for expression. Thus, in this embodiment, a plasmid containing the encoding sequence which is capable of being transformed into competent *H. influenzae htrB* mutants is introduced by methods for transformation known in the art (Karudapuram et al., 1995, *J. Bacteriol.* 177:3235-3240; Setlow et al., 1981, *supra*, herein incorporated by reference). The encoding sequence may then be recombined into the *H. influenzae htrB* mutants' genome where it is expressed under the control of its own promoter or an *H. influenzae* promoter near the site of insertion. Such transformation is reported to be at a relatively high frequency (McCarthy and Cox, 1986, *J. Bacteriol.*, 168:186-191).

Alternatively, transformation of competent *H. influenzae htrB* mutants by a circular plasmid with the appropriate origin(s) of replication and containing the encoding sequence may result in plasmid establishment; i.e., a plasmid coexisting as an extrachromosomal element without recombination. Examples of such plasmids have been described above. Thus, in this variation of the embodiment, a plasmid containing the encoding sequence which is capable of being transformed into, and established in, competent *H. influenzae htrB* mutants is introduced by methods for transformation known in the art. The encoding sequence is then expressed from the plasmid under the control of its own promoter or a promoter within the vector.

Electroporation of non-competent bacterial cells

Yet another mechanism to introduce a recombinant DNA molecule containing the encoding sequence to be expressed into at least one heterologous bacterial antigens, is to introduce a circular vector, such as a plasmid having inserted into it the recombinant DNA molecule containing the encoding sequence to be expressed, into non-competent host *H. influenzae htrB* mutants by electroporation. Electroporation has been used to efficiently introduce plasmid DNA into bacteria. However, optimal conditions may differ depending on the host cell used. Optimal conditions have been described for electroporating plasmid DNA into *H. influenzae* (Mitchell et al., 1991, *Nucl. Acids Res.* 19:3625-3628; herein incorporated by reference). It was found that electroporation of plasmid into *H. influenzae* made competent by defined, nutrient poor media was several orders of magnitude less efficient than electroporation into non-competent *H. influenzae*. Thus, in this variation of the embodiment, it would be preferred that a plasmid containing the encoding sequence is electroporated into non-competent *H. influenzae htrB* mutants. The plasmid is capable of being established in *H. influenzae htrB* mutants, or is degraded after the encoding sequence has recombined into the *H. influenzae htrB* mutants' genome. In either case, the encoding sequence is under the control of its own promoter; or a promoter within the vector or genome, respectively.

EXAMPLE 6

Neisserial htrB mutants as immunogens

In another embodiment, the *htrB* mutant is a *Neisserial htrB* mutant selected from the group including *Neisseria gonorrhoeae*, and *Neisseria meningitidis*. *N. gonorrhoeae* is a gram-negative bacterial pathogen

causing the sexually transmitted disease gonorrhea, which subsequently can lead to pelvic inflammatory disease in females. *N. meningitidis* is a gram-negative bacterial pathogen which can cause a variety of clinical infections including bacteremia, septicemia, meningitis, and pneumonia. Alterations in the terminal glycosylation of the LOS of *Neisseria* are believed correlate with serum sensitivity and serum resistance of the organism. Further, protective bactericidal antibody is directed against type-specific antigens of *N. meningitidis*, wherein the type-specific antigens have been identified as outer membrane proteins, or LOS, or both.

Using the methods according to the present invention, as illustrated in Examples 1-3 and 10, *htrB* mutants of a *Neisserial* species can be produced and identified. One skilled in the art, using the *htrB* gene of *H. influenzae*, can isolate the *htrB* gene of the *Neisserial* species, and produce a mutated *htrB* gene (unable to encode functional HtrB) using transposon mutagenesis with subsequent recombination resulting in a *Neisserial htrB* mutant lacking one or more secondary acyl chains. Alternatively, there may be sufficient homology between *Neisseria* and *Haemophilus* to use plasmids pB28 and pB29, each with a mini-Tn3 transposon containing the chloramphenicol acetyltransferase (CAT) gene inserted into the *htrB* open reading frame at a different location, to transform the *Neisserial* species for recombination of the mutant *htrB* gene into the *Neisserial htrB* gene. *Neisserial* transformants are then selected for by growth in the presence of chloramphenicol (1.5 μg/ml), resulting in identification of *Neisserial htrB* mutant strains. Locations of the mTn3 insertion in the chromosomes of the *Neisserial htrB* mutants may be confirmed by genomic Southern hybridization using a probe containing *htrB* sequences.

The resultant *Neisserial htrB* mutants can then be tested for substantially reduced toxicity using assays described by those skilled in the art for measuring the toxic effects induced by endotoxin.

Using the methods according to the present invention, as illustrated in Examples 4 & 5, endotoxin isolated from a *Neisserial htrB* mutant can be used in a vaccine formulation in inducing immunity against the wild type strains of *Neisserial* pathogens. The *htrB* mutant LOS may be isolated by a method known to those skilled in the art for isolating LOS. The *htrB* mutant LOS may be used in a vaccine formulation containing one or more agents selected from the group consisting of a pharmaceutically acceptable carrier (e.g., a physiological solution), an adjuvant, or a carrier protein.

Alternatively, *Neisserial htrB* mutants can be used in a live bacterial vaccine preparation, in an inactivated bacterial vaccine preparation, and can be genetically engineered to express at least one heterologous bacterial antigen in a multivalent vaccine preparation. Regarding the latter aspect, plasmids useful for cloning of and expression from recombinant DNA molecules into *Neisserial* species are known to those skilled in the art, including:

pLES2 confers ampicillin resistance, is a shuttle vector functional in both *E. coli* and *N. gonorrhoeae*, and contains a polylinker with restriction sites for *Eco*RI, *Sma*I, and *Bam*HI (Stein et al., 1983, *Gene* 25:241-247). *Neisserial* species also contain a natural transformation process (Rudel et al., 1995, *Proc Natl Acad Sci USA* 92:7896-90; Goodman et al., 1991, *J Bacteriol* 173:5921-5923); and can also be made competent or be electroporated using techniques known to those skilled in the art.

EXAMPLE 7

Haemophilus ducreyi htrB mutants as immunogens

In another embodiment, the mutant is a *H. ducreyi htrB* mutant. *H. ducreyi* is a gram-negative bacterial pathogen causing a genital ulcer disease, chancroid. Using the methods according to the present invention, as illustrated in Examples 1-3 and 10, *H. ducreyi htrB* mutants can be produced and identified. One skilled in the art, using the *htrB* gene of *H. influenzae*, can isolate the *htrB* gene of *H. ducreyi*, and produce a mutated *htrB* gene (unable to encode functional HtrB) using transposon mutagenesis with subsequent recombination resulting in an *H. ducreyi htrB* mutant lacking one or more secondary acyl chains. Alternatively, there is likely sufficient homology between *H. ducreyi* and *H. influenzae* to use plasmids pB28 and pB29, each with a mini-Tn3 transposon containing the chloramphenicol acetyltransferase (CAT) gene inserted into the *htrB* open reading frame at a different location, to transform *H. ducreyi* for recombination of the mutant *htrB* gene into the *H. ducreyi htrB* gene. *H. ducreyi* transformants are then selected for by growth in the presence of chloramphenicol (1.5 μg/ml), resulting in identification of *H. ducreyi htrB* mutant strains. Locations of the mTn3 insertion in the chromosomes of the *H. ducreyi htrB* mutants may be confirmed by genomic Southern hybridization using a probe containing *htrB* sequences. The resultant *H. ducreyi htrB* mutants can then be tested for substantially reduced toxicity using assays described by those skilled in the art for measuring the toxic effects induced by endotoxin.

Using the methods according to the present invention, as illustrated in Examples 4 & 5, endotoxin isolated from an *H. ducreyi htrB* mutant can be used in a vaccine formulation in inducing immunity against the wild type strains of *H. ducreyi*. The *htrB* mutant LOS

may be isolated by a method known to those skilled in the art for isolating LOS. The *htrB* mutant LOS may be used in a vaccine formulation containing one or more agents selected from the group consisting of a pharmaceutically acceptable carrier (e.g., a physiological solution), an adjuvant, or a carrier protein.

Alternatively, *H. ducreyi htrB* mutants can be used in a live bacterial vaccine preparation, in an inactivated bacterial vaccine preparation, and can be genetically engineered to express at least one heterologous bacterial antigen in a multivalent vaccine preparation. Regarding the latter aspect, plasmids useful for cloning of and expression from recombinant DNA molecules into *Haemophilus* species are known to those skilled in the art, as disclosed in Example 5; and can also be made competent or be electroporated using techniques known to those skilled in the art, as disclosed in Example 5.

EXAMPLE 8

Campylobacter jejuni htrB mutants as immunogens

In another embodiment, the mutant is a *C. jejuni htrB* mutant. *Campylobacter jejuni* is a gram-negative bacterial pathogen causing human enteritis. Infection by *C. jejuni* has also been associated with the onset of neurologic disorders such as Guillian-Barré syndrome. *C. jejuni htrB* mutants can be produced and identified using the methods according to the present invention, as illustrated in Examples 1-3 and 10. One skilled in the art, using the *htrB* gene of *H. influenzae*, can isolate the *htrB* gene of *C. jejuni*, and produce a mutated *htrB* gene (unable to encode functional HtrB) using transposon mutagenesis with subsequent recombination resulting in an *C. jejuni htrB* mutant lacking one or more secondary acyl chains.

Alternatively, there may be sufficient homology between *C. jejuni* and *H. influenzae* to use plasmids pB28 and pB29, each with a mini-Tn3 transposon containing the chloramphenicol acetyltransferase (CAT) gene inserted into the *htrB* open reading frame at a different location, to transform *C. jejuni* for recombination of the mutant *htrB* gene into the *C. jejuni htrB* gene. *C. jejuni* transformants are then selected for by growth in the presence of chloramphenicol (1.5 μg/ml), resulting in identification of *C. jejuni htrB* mutant strains. Locations of the mTn3 insertion in the chromosomes of the *C. jejuni htrB* mutants may be confirmed by genomic Southern hybridization using a probe containing *htrB* sequences. The resultant *C. jejuni htrB* mutants can then be tested for substantially reduced toxicity using assays described by those skilled in the art for measuring the toxic effects induced by endotoxin.

Using the methods according to the present invention, as illustrated in Examples 4 & 5, endotoxin isolated from a *C. jejuni htrB* mutant can be used in a vaccine formulation in inducing immunity against the wild type strains of *C. jejuni*. The *htrB* mutant LPS may be isolated by a method known to those skilled in the art for isolating LPS. The *htrB* mutant LPS may be used in a vaccine formulation containing one or more agents selected from the group consisting of a pharmaceutically acceptable carrier (e.g., a physiological solution), an adjuvant, or a carrier protein.

Alternatively, *C. jejuni htrB* mutants can be used in a live bacterial vaccine preparation, in an inactivated bacterial vaccine preparation, and can be genetically engineered to express at least one heterologous bacterial antigen in a multivalent vaccine preparation. Regarding the latter aspect, plasmids useful for cloning of and expression from recombinant

DNA molecules into *C. jejuni* are known to those skilled in the art, and includes:

pUA466 confers tetracycline resistance, and contains an unique *Ava*I site and *Ava*II site (Taylor, 1986, *J Bacteriol* 165:1037-39).

C. jejuni can also be made competent or be electroporated using techniques known to those skilled in the art.

EXAMPLE 9

Moraxella catarrhalis htrB mutants as immunogens

In another embodiment, the mutant is a *M. catarrhalis htrB* mutant. *Moraxella catarrhalis* is a gram-negative bacterial pathogen causing otitis media in children; sinusitis and conjunctivitis in children and adults; and lower respiratory tract infections, septicemia, and meningitis in immunocompromised hosts. *M. catarrhalis htrB* mutants can be produced and identified using the methods according to the present invention, as illustrated in Examples 1-3 and 10. One skilled in the art, using the *htrB* gene of *H. influenzae*, can isolate the *htrB* gene of *M. catarrhalis*, and produce a mutated *htrB* gene (unable to encode functional HtrB) using transposon mutagenesis with subsequent recombination resulting in an *M. catarrhalis htrB* mutant lacking one or more secondary acyl chains. Alternatively, there may be sufficient homology between *M. catarrhalis* and *H. influenzae* to use plasmids pB28 and pB29, each with a mini-Tn3 transposon containing the chloramphenicol acetyltransferase (CAT) gene inserted into the *htrB* open reading frame at a different location, to transform *M. catarrhalis* for recombination of the mutant *htrB* gene into the *M. catarrhalis htrB* gene. *M. catarrhalis* transformants are then selected for by growth in the presence of chloramphenicol (1.5 μg/ml), resulting in identification of *M. catarrhalis*

htrB mutant strains. Locations of the mTn3 insertion in the chromosomes of the *M. catarrhalis htrB* mutants may be confirmed by genomic Southern hybridization using a probe containing *htrB* sequences. The resultant *M. catarrhalis htrB* mutants can then be tested for substantially reduced toxicity using assays described by those skilled in the art for measuring the toxic effects induced by endotoxin.

Using the methods according to the present invention, as illustrated in Examples 4 & 5, endotoxin isolated from a *M. catarrhalis htrB* mutant can be used in a vaccine formulation in inducing immunity against the wild type strains of *M. catarrhalis*. The *htrB* mutant LOS may be isolated by a method known to those skilled in the art for isolating LPS. The *htrB* mutant LOS may be used in a vaccine formulation containing one or more agents selected from the group consisting of a pharmaceutically acceptable carrier (e.g., a physiological solution), an adjuvant, or a carrier protein.

Alternatively, *M. catarrhalis htrB* mutants can be used in a live bacterial vaccine preparation, in an inactivated bacterial vaccine preparation, and can be genetically engineered to express at least one heterologous bacterial antigen in a multivalent vaccine preparation. Regarding the latter aspect, plasmids useful for cloning of and expression from recombinant DNA molecules into *M. catarrhalis* are known to those skilled in the art. *M. catarrhalis* contains a natural transformation process (Juni, 1977, *J Clin Microbiol* 5:227-35) and can also be made competent or be electroporated using techniques known to those skilled in the art.

EXAMPLE 10

Salmonella htrB mutants as immunogens

In another embodiment, the mutant is a *Salmonella htrB* mutant. *Salmonella* species comprise gram-negative bacteria that can cause a variety of clinical illnesses in humans and animals. For example, *S. typhi* is the causative agent of typhoid fever in humans. *S. paratyphi* is a causative organism of a fever known as salmonella fever in humans. Salmonellosis, a gastroenteritis in humans, can be caused by various species in the genus *Salmonella* (*typhimurium, newport, heidelberg,* and *enteritidis*). *Salmonella htrB* mutants can be produced and identified. One skilled in the art, using the *htrB* gene of a gram-negative bacterial pathogen, can produce a mutated *htrB* gene (unable to encode functional HtrB) using transposon mutagenesis and subsequeent recombination, ultimately resulting in an *Salmonella htrB* mutant having a modification in one or more secondary acyl chains. The resultant *Salmonella htrB* mutants can then be tested for substantially reduced toxicity using assays described by those skilled in the art for measuring the toxic effects induced by endotoxin.

To illustrate this embodiment, and using methods similar to those in Example 1 herein, mutagenesis of the *htrB* gene was carried out by shuttle mutagenesis by mini-Tn10 (conferring tetracycline resistance) used as an insertion sequence to mutate the *htrB* gene. The *htrB*:Tn10 was then transfered from *E. coli* to a virulent *S. typhimurium* by transduction. Using methods previously described (Masters, 1996, in E. coli and Salmonella Cellular and Molecular Biology, vol.2, 2nd edition, p. 2421, ASM Press), a r^-m^+ *galE mutS recD S. typhimurium* (SL5283) was sequentially transduced with MST3488 (*recD*542:Tn10d which confers chloramphenicol resistance (cm^r)) via *Salmonella* phage P22 resulting in a r^-m^+ galE mutS recD cm^r *S. typhimurium* ("MGS-1"), and then with MST3063 (*mutS*:Tn10 which

confers tertracycline resistant (tet^r)) resulting in a r$^-$ m$^+$ galE mutS recD cm^r tet^r *S. typhimurium* ("MGS-3"). *S. typhimurium* MGS-3 was cured of Tn10 by selection for tetracycline sensitivity on media using methods previously described (Bochner et al., 1990, *J. Bacteriol.* 143:926-933) resulting in a r$^-$m$^+$ *galE mutS recD* cm^r *S. typhimurium* ("MGS-7"). For confirmation purposes, it was shown that *S. typhimurium* MGS-7 showed the same response to ultraviolet light as the parental strain MGS-3.

An *E. coli* strain ("MLK217") containing the *htrB*:mini Tn10 was used to transfer the *htrB*:Tn10 by transduction into *S. typhimurium* MGS-7 via coliphage P1 according to methods previously described (Masters, 1996, *supra*), and selected for by growth at 30°C on media plates containing tetracycline. The result of the transduction, and after reisolation for tetracycline-resistant clones, was the creation of a r$^-$m$^+$ *galE mutS recD htrB*:mini Tn10 cm^r tet^r *S. typhimurium* ("MGS-23"). *S. typhimurium* MGS-23 was tested for one or more of the phenotypic properties associated with *htrB* mutation, namely (1) temperature sensitivity; (2) filamentation and bulging at non-permissive temperatures; and (3) deoxycholate resistance. The results of the phenotypic analysis indicated that MGS-23 carried the miniTn 10 element inserted within the *S. typhimurium htrB* gene because MGS-23 was able to grow at 30°C but not at 37°C; formed many filamentous forms when shifted to non-permissive temperature; and showed resistance to higher concentrations of deoxycholate (7.5% to 10%) than the isogenic parent (2.5%). The mutation of the *htrB* was further confirmed by analysis using polymerase chain reaction.

A virulent *S. typhimurium* strain (SL1344) was transduced to *htrB:*Tn10 from *S. typhimurium* MGS-23 via *Salmonella* phage P22 and selection at 30°C on media

plates containing tetracycline. After isolation, resultant tetracycline resistant clones having the same phenotype as MGS-23 were further analyzed. One such clone, MGS-31, was shown to have a mutated *htrB* gene, by complementing the clone using a plasmid with a wild type *htrB* gene (Karow et al., 1991, *J. Bacteriol.* 173:741-50; Karow et al., 1991, *Mol. Microbiol.* 5:2285-2292) thereby returning the clone to the wild type phenotype of normal growth, normal cell morphology, deoxycholate sensitivity at 37°C, and wild type virulence.

Endotoxin characteristics

Mass spectrometry was used to analyze the lipid A according to the methods in Example 2 herein. More specifically, lipid A from *S. typhimurium htrB* mutant LPS and from wild type *S. typhimurium* LPS were each analyzed by liquid secondary ion mass spectrometry (LSIMS) in the negative ion mode to provide a spectrum of molecular ions for the different components lipid A. The chemical analysis of the lipid A of the *S. typhimurium htrB* mutant indicated that the modifications in the lipid A structure that occurred were similar, but not identical, to modifications in the lipid A structure seen in *H. influenzae htrB* mutants. In the wild type *S. typhimurium* lipid A contains either six (hexaacyl) or seven (heptaacyl) fatty acid substitutions from the diglucosamine backbone (FIG. 7). In the wild type strain, on glucosamine II, the 3' substitution on the N-linked C14 fatty acid (hexaacyl or heptaacyl) is a C12 fatty acid. In contrast, and as shown in FIG. 7, the C12 fatty acid is replaced with a C16 fatty acid. These results indicate that the *S. typhimurium htrB* gene encodes an acyltransferase responsible for placing the C12 fatty acid at the 3' position on the N-linked C14 fatty acid. Mutation of the *S. typhimurium htrB* gene results in the functional induction of another acyltransferase which places a C16 fatty acid at the 3'

position on the N-linked C14 fatty acid. It is known to those skilled in the art, that lipid A is crucial for the survival of a gram-negative organism, and for the proper organization of its outer membrane. Thus, and as related to virulence and toxicity of the organism, the effects of the *htrB* gene mutation in *S. typhimurium* was analyzed.

Endotoxin toxicity

A mouse model system that is used by those skilled in the art as relevant to human disease, is the D-galactosamine model. In the D-galactosamine model, the sensitivity to LPS is increased by exposure to D-galactosamine (Galanos et al., 1986, *Infect. Immun.* 51:891-896) thereby achieving the same lethality and TNF induction with low doses of LPS, i.e. with levels of endotoxin commensurate with those identified in the blood of septic patients. Thus, D-galactosamine-treated mice exposed to LPS is a standard animal model system accepted by those skilled in the art as relevant to endotoxic shock in humans. In this model, groups of 4 mice were treated with D-galactosamine (8 mg) simultaneously with the administration of the dosage of the LPS to be tested. Groups of 4 mice each were injected with either 0.001 μg, 0.01 μg, 0.1 μg, 1 μg or 10 μg of the purified LPS to be tested and the number of mice surviving the challenge at each dose were checked every 24 hours for 5 days after the injection. The LD_{50} (lethal dose where 50% of the mice are killed) is then calculated. The purified LPS to be separately tested included LPS from the wild type virulent *S. typhimurium* strain 1344; and the *S. typhimurium htrB* mutant (MGS-31). The results show that the LD_{50} for mice injected with LPS from *S. typhimurium* strain 1344 is 0.01 μg. In contrast, the LD_{50} for mice injected with LPS from *S. typhimurium htrB* mutant MGS-31 is 0.1 μg. Thus, the lipid A from the *S. typhimurium htrB* mutant is at least

10 fold less toxic than the lipid A of the wild type strain.

Virulence

Since mice are naturally susceptible to infection by *S. typhimurium*, like humans, a second mouse model was used to assess the effects of the *htrB* mutation on virulence of *S. typhimurium*. Typically, from approximately 50 cfu to 100 cfu will kill 50 to 100% of the mice injected. The strains used included *S. typhimurium* strain 1344; *S. typhimurium htrB* mutant (MGS-31); and the *S. typhimurium htrB* mutant which was complemented by the plasmid containing an intact *htrB* gene (MGS-43). The respective organisms were injected intraperitoneally in a dosage ranging from 5×10^1 to 5×10^7 cfu and watched over 5 days. Generaly, 100% of the animals who will die from bacteremia, do so within that period. As may be expected, the LD_{50} for the virulent *S. typhimurium* strain 1344 was less than 5×10^1 cfu. Likewise, the LD_{50} for the *S. typhimurium htrB* mutant which was complemented with the intact *htrB* gene, MGS-43, also was less than 5×10^1 cfu. In contrast, the LD_{50} for the *S. typhimurium htrB* mutant MGS-31 was 9.7×10^6 cfu, an approximately 2×10^5 reduction in virulence compared to the wild type virulent strain. *S. typhimurium htrB* mutant growth in vivo in mice was confirmed by culturing and assaying liver and spleen for bacterial counts. The results suggest that the *htrB* mutation in *Salmonella* has a more profound effect on virulence factors than just a modification of the LPS.

Using the methods according to the present invention, as illustrated in Examples 4 & 5, endotoxin isolated from a *Salmonella htrB* mutant can be used in a vaccine formulation in inducing immunity against the wild type strains of the *Salmonella* species. The *htrB* mutant LPS may be isolated by a method known to those skilled in the art for isolating LPS. The *htrB* mutant

LPS may be used in a vaccine formulation containing one or more agents selected from the group consisting of a pharmaceutically acceptable carrier (e.g., a physiological solution), an adjuvant, or a carrier protein.

Alternatively, *Salmonella htrB* mutants can be used in a live bacterial vaccine preparation, in an inactivated bacterial vaccine preparation, and can be genetically engineered to express at least one heterologous bacterial antigen in a multivalent vaccine preparation. Regarding the latter aspect, plasmids useful for cloning of and expression from recombinant DNA molecules into *Salmonella* are known to those skilled in the art, and includes:

pYA260 containing *lacZ* cloned into a *trc* promoter; and **pJW270** conferring tetracycline resistance and containing *lacI* (Ervin et al., 1993 *Microb Pathogen* 15:93-101).

pB7 confers kanamycin and chloramphenicol resistance, and contains a cloning site flanked by a *Bal*I site and a *Hind*III site (Purcell et al., 1983, *Infect Immun* 39:1122-1127).

pACK5 contains the replicon of pAC1 from *Acetobacter pasteurianus* and confers kanamycin resistance (Grones et al., 1995, *Biochem Biophys Res Commun* 206:942-947).

pVAC468 is a suicide vector for chromosomal insertion of heterologous antigens into *Salmonella* and contains a polylinker having the following restriction sites: *Cla*I, *Eco*RV, *Xho*I, *Sac*I, *Sal*I, *Sma*I, *Xba*I, and *Bgl*II (Hohmann et al., 1995, *Proc Natl Acad Sci USA* 92:2904-2908).

Also disclosed is a bacteriophage system, a 'chromosomal expression vector' for inserting genes encoding foreign antigens into the chromosome of *Salmonella*, which uses a defective transposable element carried on bacteriophage lambda (Flynn et al., 1990, *Mol Microb* 4:2111-2118).

Salmonella can also be made competent (see for example, Purcell et al., 1983, *supra*) or be electroporated using

techniques known to those skilled in the art (see for example, Grones et al., 1995, *supra*; Coulson et al., 1994, *supra*).

EXAMPLE 11

Shigella htrB mutants as immunogens

In another embodiment, the mutant is a *Shigella* species *htrB* mutant. Members of the genus *Shigella* are gram-negative bacteria which cause diseases such as dysentery (pathogenic species include *dysenteriae*, *sonnei*, and *flexneri*) primarily in humans. *Shigella htrB* mutants can be produced and identified using the methods according to the present invention, as illustrated in Examples 1-3 and 10. One skilled in the art, using the *htrB* gene of *H. influenzae*, can isolate the *htrB* gene of *Shigella*, and produce a mutated *htrB* gene (unable to encode functional HtrB) using transposon mutagenesis with subsequent recombination resulting in an *Shigella htrB* mutant lacking one or more secondary acyl chains. Alternatively, there may be sufficient homology between *Shigella* and *H. influenzae* to use plasmids pB28 and pB29, each with a mini-Tn3 transposon containing the chloramphenicol acetyl-transferase (CAT) gene inserted into the *htrB* open reading frame at a different location, to transform *Shigella* for recombination of the mutant *htrB* gene into the *Shigella htrB* gene. *Shigella* transformants are then selected for by growth in the presence of chloramphenicol (1.5 μg/ml), resulting in identification of *Shigella htrB* mutant strains. Locations of the mTn3 insertion in the chromosomes of the *Shigella htrB* mutants may be confirmed by genomic Southern hybridization using a probe containing *htrB* sequences. The resultant *Shigella htrB* mutants can then be tested for substantially reduced toxicity using assays described by those skilled in the art for measuring the toxic effects induced by endotoxin.

Using the methods according to the present invention, as illustrated in Examples 4 & 5, endotoxin isolated from an *htrB* mutant made from a pathogenic *Shigella* species can be used in a vaccine formulation in inducing immunity against the wild type strains of *Shigella*. The *htrB* mutant LPS may be isolated by a method known to those skilled in the art for isolating LPS. The *htrB* mutant LPS may be used in a vaccine formulation containing one or more agents selected from the group consisting of a pharmaceutically acceptable carrier (e.g., a physiological solution), an adjuvant, or a carrier protein.

Alternatively, *Shigella htrB* mutants can be used in a live bacterial vaccine preparation, in an inactivated bacterial vaccine preparation, and can be genetically engineered to express at least one heterologous bacterial antigen in a multivalent vaccine preparation. Regarding the latter aspect, plasmids useful for cloning of and expression from recombinant DNA molecules into *Shigella* are known to those skilled in the art, and includes:

pACK5 contains the replicon of pAC1 from *Acetobacter pasteurianus* and confers kanamycin resistance (Grones et al., 1995, *supra*).

Shigella can also be made competent or be electroporated using techniques known to those skilled in the art.

EXAMPLE 12

Pseudomonas aeruginosa htrB mutants as immunogens

In another embodiment, the mutant is a *Pseudomonas aeruginosa htrB* mutant. *Pseudomonas aeruginosa* is a gram-negative bacterial pathogen which cause diseases such as respiratory tract infections and sepsis, particularly in immunocompromised patients. Other pathogenic species for humans and animals include *pseudomallei*, and *mallei*. Mass spectrometry and nuclear

magnetic resonance spectroscopy were used to determine the structure of lipid A of *Pseudomonas aeruginosa* LPS. The structure of *P. aeruginosa* lipid A was found to be the same as *Enterobacterial* lipid A: a backbone of a glucosamine disaccharide which is either mono-phosphorylated or diphosphorylated (positions 1 and 4'); and which carries several molecules of ester- and amide-bound fatty acids. In addition to the hexaacyl and pentaacyl lipid A species, a tetraacyl species was identified (Karunaratne et al., 1992, *Arch Biochem Biophys* 299:368-76).

Pseudomonas htrB mutants (e.g., *P. aeruginosa*) can be produced and identified using the methods according to the present invention, as illustrated in Examples 1-3 and 10. One skilled in the art, using the *htrB* gene of *H. influenzae*, can isolate the *htrB* gene of *Pseudomonas aeruginosa*, and produce a mutated *htrB* gene (unable to encode functional HtrB) using transposon mutagenesis with subsequent recombination resulting in a *P. aeruginosa htrB* mutant lacking one or more secondary acyl chains. Alternatively, there may be sufficient homology between *P. aeruginosa* and *H. influenzae* to use plasmids pB28 and pB29, each with a mini-Tn3 transposon containing the chloramphenicol acetyltransferase (CAT) gene inserted into the *htrB* open reading frame at a different location, to transform *P. aeruginosa* for recombination of the mutant *htrB* gene into the *P. aeruginosa htrB* gene. *P. aeruginosa* transformants are then selected for by growth in the presence of chloramphenicol (1.5 μg/ml), resulting in identification of *P. aeruginosa htrB* mutant strains. Locations of the mTn3 insertion in the chromosomes of the *P. aeruginosa htrB* mutants may be confirmed by genomic Southern hybridization using a probe containing *htrB* sequences. The resultant *P. aeruginosa htrB* mutants can then be tested for substantially reduced toxicity using assays

described by those skilled in the art for measuring the toxic effects induced by endotoxin.

Using the methods according to the present invention, as illustrated in Examples 4 & 5, endotoxin isolated from a *P. aeruginosa htrB* mutant can be used in a vaccine formulation in inducing immunity against the wild type strains of *P. aeruginosa*. The *htrB* mutant LPS may be isolated by a method known to those skilled in the art for isolating LPS. The *htrB* mutant LPS may be used in a vaccine formulation containing one or more agents selected from the group consisting of a pharmaceutically acceptable carrier (e.g., a physiological solution), an adjuvant, or a carrier protein.

Alternatively, *P. aeruginosa htrB* mutants can be used in a live bacterial vaccine preparation, in an inactivated bacterial vaccine preparation, and can be genetically engineered to express at least one heterologous bacterial antigen in a multivalent vaccine preparation. Regarding the latter aspect, plasmids useful for cloning of and expression from recombinant DNA molecules into *P. aeruginosa* are known to those skilled in the art, and includes:

pPAH121 confers carbenicillin resistance, and contains a unique *Hpa*I restriction site (Hoyne et al., 1992, *J Bacteriol* 174:7321-7327.

P. aeruginosa can also be made competent (see for example, Hoyne et al., 1992, *supra*) or be electroporated using techniques known to those skilled in the art.

EXAMPLE 13

Multivalent *htrB* mutant vaccine formulation

In one embodiment according to the present invention, as illustrated in Examples 4 & 5, the *htrB* mutant is genetically engineered to express one or more heterologous microbial antigens in producing a

multivalent vaccine using methods known to those skilled in the art. In a preferred embodiment, a microbial pathogen may include a respiratory pathogen selected from the group of pathogens, with respective antigens, in Table 2.

Table 2

PATHOGEN	INFECTION/DISEASE	PROTEIN ANTIGEN
H. influenzae	otitis media, lower respiratory tract	D-15, P1, P6[1]
Group A Streptococcus	pharyngitis, rheumatic fever	M[2]
Branhamella catarrhalis	otitis media, lower respiratory tract	CD, E[3]
Streptococcus pneumoniae	pneumonia, otitis media, meningitis	autolysin, pneumolysin[4]
Bordetella pertussis	pertussis (whooping cough)	filamentous hem-agglutinin, pertussis toxin, 69kDa Omp[5]
Pseudomonas aeruginosa	respiratory tract	Omp OprF, exotoxin A[6]
Legionella pneumophila	pneumonia	OmpS, Hsp60[7]
Mycoplasma pneumoniae	upper and lower respiratory tract	P1[8]
Respiratory syncytial virus	lower respiratory tract	M2, P, F, G[9]
Influenza virus	influenza	HA, M[10]
Adenovirus	common cold	
rhinovirus	common cold	VP1, VP2, VP3[11]
Parainfluenza virus	common cold	HN, F[12]
Pneumocystis carinii	pneumonia in AIDS	msg[13]

[1]- (Flack et al., 1995 *Gene* 156:97-99; Panezutti et al., 1993, 61:1867-1872; Nelson et al., 1988, *Rev Infect Diseases* 10:S331-336).
[2]- (Pruksakorn et al., 1994, *Lancet* 344:639-642; Dole et al., 1993, *J Immunol* 151:2188-94).
[3]- (Murphy et al., 1989, *Infect Immun* 57:2938-2941; Faden et al., 1992, *Infect Immun* 60:3824-3829).
[4]- (Lock et al., 1992, *Microb Pathog* 12:137-143).
[5]- (Novotny et al., 1991, *Dev Biol Stand* 73:243-249; Lipscombe et al., 1991, *Mol Microbiol* 5:1385-1392; He et al., 1993, *Eur J Clin Microbiol Infect Dis* 12:690-695).
[6]- (Rawling et al., 1995, *Infect Immun* 63:38-42; Pennington et al., 1988, *J Hosp Infect* 11A:96-102).
[7]- (Weeratna et al., 1994, *Infect Immun* 62:3454-3462).
[8]- (Jacobs et al., 1990, *Infect Immun* 58:2464-2469; 1990, *J Clin Microbiol* 28:1194-1197).
[9]- (Kulkarni et al., 1995, *J Virol* 69:1261-1264; Leonov et al., 1994, *J Gen Virol* 75:1353-1359; Garcia et al., 1993, *Virology* 195:239-242; Vaux-Peretz et al., 1992, *Vaccine* 10:113-118).
[10]- (Kaly et al., 1994, *Vaccine* 12:753-760; Bucher et al., 1980, *J Virol* 36:586-590).
[11]- (Francis et al., 1987, *J Gen Virol* 68:2687-2691).
[12]- (Morein et al., 1983, *J Gen Virol* 64:1557-1569).
[13]- (Garbe et al., 1994, *Infect Immun* 62:3092-3101).

In another preferred embodiment, a microbial pathogen may include a pathogen causing a sexually transmitted disease selected from the group of pathogens, with respective antigens, in Table 3.

Table 3

PATHOGEN	INFECTION/DISEASE	PROTEIN ANTIGEN
N. gonorrhoeae	gonorrhea	IgA1 protease[1], PIB[2], H.8[3], Por[4]
Chlamydia trachomatis	nongonococcal urethritis	MOMP[5], HSP[6]

[1]- (Lomholt et al., 1994, *Infect Immun* 62:3178-83).
[2]- (Heckels et al., 1990, *Vaccine* 8:225-230).
[3]- (Blacker et al., 1985, *J Infect Dis* 151:650-657).
[4]- (Wetzler et al., 1992, *Vaccine* 8:225-230).
[5]- (Campos et al., 1995, *Ophthamol Vis Sci* 36:1477-91; Murdin et al., 1995, *Infect Immun* 63:1116-21).
[6]- (Taylor et al., 1990, *Infect Immun* 58:3061-3).

Tables 2 & 3, and the references footnoted which are herein incorporated by reference, illustrate various protein antigens, or peptides thereof, viewed by those skilled in the art to be useful as vaccine candidates against the respective microbial pathogen. Typically, the immunopotency of an epitope, whether from a protein or peptide, of a microbial pathogen is determined by monitoring the immune response of an animal following immunization with the epitope and/or by analyzing human convalescent sera in conjunction with pre-immune sera. Thus, one skilled in the art can determine protein or peptide antigens from microbial pathogens which would be desired to include as a heterologous antigen to be expressed by an *htrB* mutant according to the present invention. A corresponding nucleic acid sequence, the encoding sequence, can then be deduced from the amino acid sequence of the protein or peptide antigen, wherein the encoding sequence is introduced into the *htrB* mutant for expression.

EXAMPLE 14

Use of *htrB* mutants to generate antisera

The *htrB* mutant, or endotoxin purified therefrom, can be used to generate endotoxin-specific antisera, directed to the particular gram-negative bacterial pathogen, which can be used in an immunoassay to detect the antigen (that particular gram-negative bacterial pathogen), present in the body fluid of an individual suspected of having an infection caused by that gram-negative bacterial pathogen. The body fluid(s) collected for analysis depend on the microorganism to be detected, the suspected site of infection, and whether the body fluid is suspected of containing the antigen or containing antisera. With those considerations in mind, the body fluid could include one or more of sputum, blood, cerebrospinal

fluid, lesion exudate, swabbed material from the suspected infection site, and fluids from the upper respiratory tract. Immunoassays for such detection comprises any immunoassay known in the art including, but not limited to, radioimmunoassay, ELISA, "sandwich" assay, precipitin reaction, agglutination assay, fluorescent immunoassay, and chemiluminescence-based immunoassay.

Alternatively, where an immunocompromised individual is suffering from a potentially life-threatening infection caused by a particular gram-negative bacterial pathogen, immunization may be passive, i.e. immunization comprising administration of purified human immunoglobulin containing antibody against an *htrB* mutant or isolated *htrB* endotosin of that particular gram-negative bacterial pathogen.

It should be understood that while the invention has been described in detail herein, the examples were for illustrative purposes only. Other modifications of the embodiments of the present invention that are obvious to those skilled in the art of molecular biology, medical diagnostics, and related disciplines are intended to be within the scope of the appended claims.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

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Melvin G. Sunshine
Na-Gyong Lee
Bradley Gibson
Rasappa Arumugham

(ii) TITLE OF INVENTION: Non-Toxic Mutants of Pathogenic Gram-Negative Bacteria

(iii) NUMBER OF SEQUENCES: 5

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(F) ZIP: 14203-2391

(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Diskette, 3.5 inch, 1.4 Mb storage
(B) COMPUTER: IBM Compatible
(C) OPERATING SYSTEM: MS-DOS/ Microsoft Windows 3.1
(D) SOFTWARE: Wordperfect for Windows 5.1

(vi) APPLICATION DATA:
(A) APPLICATION NUMBER:
(B) FILING DATE:

(vii) ATTORNEY/AGENT INFORMATION:
(A) NAME: Nelson, M. Bud
(B) REGISTRATION NUMBER: 35,300
(C) REFERENCE DOCKET NUMBER: 22244.0002

(viii) TELECOMMUNICATION INFORMATION:
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(B) TELEFAX: (716) 849-0349

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 969 nucleotides
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double-stranded
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: genomic DNA

(iii) HYPOTHETICAL: yes

(iv) ORIGINAL SOURCE:
(A) ORGANISM: *H. influenzae*
(B) STRAIN: 2019
(C) CELL TYPE: bacterium

(v) SEQUENCE DESCRIPTION: SEQ ID NO:1:

TAAACTACGC CCCTAACTTA CGTGGAAAGA ACA ATG AAA AAC 42

```
                                                          Met Lys Asn
                                                            1

     GAA AAA CTC CCT CAA TTT CAA CCG CAC TTT TTA GCC CCA 81
 5   Glu Lys Leu Pro Gln Phe Gln Pro His Phe Leu Ala Pro
           5                   10                  15

     AAA TAC TGG CTT TTT TGG CTA GGC GTG GCA ATT TGG CGA 120
     Lys Tyr Trp Leu Phe Trp Leu Gly Val Ala Ile Trp Arg
10                 20                  25

     AGT ATT TTA TGT CTT CCC TAT CCT ATT TTG CGC CAT ATT 159
     Ser Ile Leu Cys Leu Pro Tyr Pro Ile Leu Arg His Ile
      30                  35                  40
15
     GGT CAT GGT TTC GGT TGG CTG TTT TCA CAT TTA AAA GTG 198
     Gly His Gly Phe Gly Trp Leu Phe Ser His Leu Lys Val
               45                  50                  55

20   GGT AAA CGT CGA GCT GCC ATT GCA CGC CGT AAT CTT GAA 237
     Gly Lys Arg Arg Ala Ala Ile Ala Arg Arg Asn Leu Glu
                         60                  65

     CTT TGT TTC CCT GAT ATG CCT GAA AAC GAA CGT GAG ACG 276
25   Leu Cys Phe Pro Asp Met Pro Glu Asn Glu Arg Glu Thr
           70                  75                  80

     ATT TTG CAA GAA AAT CTT CGT TCA GTA GGC ATG GCA ATT 315
     Ile Leu Gln Glu Asn Leu Arg Ser Val Gly Met Ala Ile
30                 85                  90                  95

     ATC GAA ACT GGC ATG GCT TGG TTT TGG TCG GAT TCA CGT 354
     Ile Glu Thr Gly Met Ala Trp Phe Trp Ser Asp Ser Arg
                        100                 105
35
     ATC AAA AAA TGG TCG AAA GTT GAA GGC TTA CAT TAT CTA 393
     Ile Lys Lys Trp Ser Lys Val Glu Gly Leu His Tyr Leu
               110                 115                 120

40   AAA GAA AAT CAA AAA GAT GGA ATT GTT CTC GTC GGC GTT 432
     Lys Glu Asn Gln Lys Asp Gly Ile Val Leu Val Gly Val
                        125                 130

     CAT TTC TTA ACG CTA GAA CTT GGC GCA CGC ATC ATT GGT 471
45   His Phe Leu Thr Leu Glu Leu Gly Ala Arg Ile Ile Gly
          135                 140                 145

     TTA CAT CAT CCT GGC ATT GGT GTT TAT CGT CCA AAT GAT 510
     Leu His His Pro Gly Ile Gly Val Tyr Arg Pro Asn Asp
50                 150                 155

     AAT CCT TTG CTT GAT TGG CTA CAA ACA CAA GGC CGT TTA 549
     Asn Pro Leu Pro Asp Trp Leu Gln Thr Gln Gly Arg Leu
     160                 165                 170
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CGC TCC AAT AAA GAT ATG CTT GAT CGT AAA GAT TTA CGC 588
Arg Ser Asn Lys Asp Met Leu Asp Arg Lys Asp Leu Arg
        175                 180                 185

GGA ATG ATC AAA GCT TTA CGC CAC GAA GAA ACC ATT TGG 627
Gly Met Ile Lys Ala Leu Arg His Glu Glu Thr Ile Trp
                190                 195

TAT GCG CCT GAT CAC GAT TAC GGC AGA AAA AAT GCC GTT 666
Tyr Ala Pro Asp His Asp Tyr Gly Arg Lys Asn Ala Val
    200                 205                 210

TTT GTT CCT TTT TTT GCA GTA CCT GAC ACT TGC ACT ACT 705
Phe Val Pro Phe Phe Ala Val Pro Asp Thr Cys Thr Thr
            215                 220

ACT GGT AGT TAT TAT TTA TTG AAA TCC TCG CAA AAC AGC 744
Thr Gly Ser Tyr Tyr Leu Leu Lys Ser Ser Gln Asn Ser
225                 230                 235

AAA GTG ATT CCA TTT GCG CCA TTA CGC AAT AAA GAT GGT 783
Lys Val Ile Pro Phe Ala Pro Leu Arg Asn Lys Asp Gly
        240                 245                 250

TCA GGC TAT ACC GTG AGC ATT TCA GCG CCT GTT GAT TTT 822
Ser Gly Tyr Thr Val Ser Ile Ser Ala Pro Val Asp Phe
                255                 260

ACA GAT TTA CAA GAT GAA GTA GCG ATA GCT GTG CGA ATG 861
Thr Asp Leu Gln Asp Glu Val Ala Ile Ala Val Arg Met
    265                 270                 275

AAT CAA ATC GTT GAA AAG GAA ATC ATG AAG GGC ATA TCA 900
Asn Gln Ile Val Glu Lys Glu Ile Met Lys Gly Ile Ser
            280                 285

CAA TAT ATG TGG CTA CAT CGT CGT TTT AAA ACA CGC CCC
Gln Tyr Met Trp Leu His Arg Arg Phe Lys Thr Arg Pro
290                 295                 300

GAT GAA AAT ACG CCT AGT TTA TAC GAT TAA  969
Asp Glu Asn Thr Pro Ser Leu Tyr Asp
        305                 310
```

(3) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 31 nucleotides
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single-stranded
- (D) TOPOLOGY: linear

(ii) ORIGINAL SOURCE:
- (A) ORGANISM: *H. influenzae*
- (B) STRAIN: 2019

(iii) IMMEDIATE SOURCE: synthesized
(iv) SEQUENCE DESCRIPTION: SEQ ID NO:2:

CCAATATGGC GCAAAATAGG ATAGGGAAGA C 31

(4) INFORMATION FOR SEQ ID NO:3:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 9 nucleotides
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single-stranded
(D) TOPOLOGY: linear
(ii) ORIGINAL SOURCE:
(A) ORGANISM: *H. influenzae*
(iii) FEATURE:
(A) OTHER INFORMATION: uptake sequence for transformation
(iv) SEQUENCE DESCRIPTION: SEQ ID NO:3:

AAGTGCGGT 9

(5) INFORMATION FOR SEQ ID NO:4:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 nucleotides
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single-stranded
(D) TOPOLOGY: linear
(ii) FEATURE:
(A) OTHER INFORMATION: hybridizes to TNFα mRNA
(iii) SEQUENCE DESCRIPTION: SEQ ID NO:4:

ATCTCTCAGC TCCACGCCAT TGGCCAGGAG 30

(6) INFORMATION FOR SEQ ID NO:5:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 nucleotides
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single-stranded
(D) TOPOLOGY: linear
(ii) FEATURE:
(A) OTHER INFORMATION: does not hybridize to TNFα mRNA
(iii) SEQUENCE DESCRIPTION: SEQ ID NO:5:

CTCCTGGCCA ATGGCGTGGA GCTGAGAGAT 30

What is claimed is:

1. A method for immunizing an individual to prevent disease caused by a gram-negative bacterial pathogen, the method comprising vaccinating the individual with a prophylactically effective amount of a vaccine formulation comprising an active ingredient selected from the group consisting of an *htrB* mutant of said gram-negative bacterial pathogen, endotoxin isolated from the *htrB* mutant of said gram-negative bacterial pathogen, endotoxin isolated from the *htrB* mutant of said gram-negative bacterial pathogen said endotoxin conjugated to a carrier protein, and an *htrB* mutant of said gram-negative bacterial pathogen which has been genetically engineered to express at least one heterologous vaccine antigen; wherein said *htrB* mutant lacks one or more secondary acyl chains of lipid A contained in the gram-negative bacterial pathogen resulting in substantially reduced toxicity when compared to lipid A of the gram-negative bacterial pathogen.

2. The method of claim 1, wherein the individual is a human, and the vaccine formulation is introduced by a route of administration selected from the group consisting of intradermal, intramuscular, intraperitoneal, intravenous, subcutaneous, ocular, intranasal, and oral administration.

3. The method of claim 2, wherein the vaccine formulation comprises an active ingredient consisting essentially of an *htrB* mutant of said gram-negative bacterial pathogen.

4. The method of claim 2, wherein the vaccine formulation comprises an active ingredient consisting essentially of endotoxin isolated from the *htrB* mutant of said gram-negative bacterial pathogen.

5. The method of claim 2, wherein the vaccine formulation comprises an active ingredient consisting essentially of endotoxin isolated from the *htrB* mutant of said gram-negative bacterial pathogen, wherein the isolated endotoxin is conjugated to a carrier protein.

6. The method of claim 2, wherein the vaccine formulation comprises an active ingredient consisting essentially of an *htrB* mutant of said gram-negative bacterial pathogen which has been genetically engineered to express at least one heterologous antigen from a microbial pathogen.

7. The method of claim 2, wherein the vaccine formulation further comprises a physiological carrier and an adjuvant.

8. The method of claim 1, wherein the individual is an animal, and the vaccine formulation is introduced by a route of administration selected from the group consisting of intradermal, intramuscular, intraperitoneal, intravenous, subcutaneous, ocular, intranasal, and oral administration.

9. The method of claim 8, wherein the vaccine formulation comprises an active ingredient consisting essentially of an *htrB* mutant of said gram-negative bacterial pathogen.

10. The method of claim 8, wherein the vaccine formulation comprises an active ingredient consisting

essentially of endotoxin isolated from the *htrB* mutant of said gram-negative bacterial pathogen.

11. The method of claim 8, wherein the vaccine formulation comprises an active ingredient consisting essentially of endotoxin isolated from the *htrB* mutant of said gram-negative bacterial pathogen, wherein the isolated endotoxin is conjugated to a carrier protein.

12. The method of claim 8, wherein the vaccine formulation comprises an active ingredient consisting essentially of an *htrB* mutant of said gram-negative bacterial pathogen which has been genetically engineered to express at least one heterologous antigen from a microbial pathogen.

13. The method according to claim 8, wherein the *htrB* mutant of said gram-negative bacterial pathogen is administered orally as an additive to animal feed.

14. The method according to claim 12, wherein the *htrB* mutant of said gram-negative bacterial pathogen which has been genetically engineered to express at least one heterologous antigen from a microbial pathogen is administered orally as an additive to animal feed.

15. The method of claim 8, wherein the vaccine formulation further comprises a physiological carrier and an adjuvant.

16. A vaccine formulation comprising an active ingredient selected from the group consisting of an *htrB* mutant of a gram-negative bacterial pathogen, endotoxin isolated from the *htrB* mutant of said gram-negative bacterial pathogen, endotoxin isolated from the *htrB* mutant of said gram-negative bacterial pathogen said

endotoxin conjugated to a carrier protein, and an *htrB* mutant of said gram-negative bacterial pathogen which has been genetically engineered to express at least one heterologous vaccine antigen; wherein said *htrB* mutant lacks one or more secondary acyl chains of lipid A contained in the gram-negative bacterial pathogen resulting in substantially reduced toxicity when compared to lipid A of the gram-negative bacterial pathogen.

17. The vaccine formulation according to claim 16, wherein the active ingredient consists essentially of an *htrB* mutant of said gram-negative bacterial pathogen.

18. The vaccine formulation according to claim 16, wherein the active ingredient consists essentially of endotoxin isolated from the *htrB* mutant of said gram-negative bacterial pathogen.

19. The vaccine formulation of claim 16, wherein the active ingredient consists essentially of endotoxin isolated from the *htrB* mutant of said gram-negative bacterial pathogen, wherein the isolated endotoxin is conjugated to a carrier protein.

20. The vaccine formulation according to claim 16, wherein the active ingredient consists essentially of an *htrB* mutant of said gram-negative bacterial pathogen which has been genetically engineered to express at least one heterologous antigen from a microbial pathogen.

21. The vaccine formulation according to claim 16, further comprising a physiological carrier and an adjuvant.

22. A method of making in a gram-negative bacterial pathogen a mutant endotoxin of substantially reduced toxicity when compared to the endotoxin of the wild type gram-negative bacterial pathogen, the method comprising mutating an *htrB* gene within the gram-negative bacterial pathogen, wherein said mutation causes a phenotype of a resultant *htrB* mutant characterized by a mutant endotoxin lacking one or more secondary acyl chains of lipid A contained in the wild type gram-negative bacterial pathogen.

23. A mutant endotoxin of substantially reduced toxicity, made according to the method of claim 22, wherein the mutant endotoxin having substantially reduced toxicity was purified from the *htrB* mutant by a process selected from the group consisting of a phenol/water extraction, and a protease digestion; and wherein the purified mutant endotoxin having substantially reduced toxicity is used to generate endotoxin-specific antibodies.

24. The mutant endotoxin according to claim 23, further comprising conjugation to a carrier protein.

25. A mutant endotoxin of substantially reduced toxicity, made according to the method of claim 22.

26. The mutant endotoxin according to claim 25, further comprising conjugation to a carrier protein.

27. A method of making an *htrB* mutant of a wild type gram-negative bacterial pathogen wherein the *htrB* mutant has substantially reduced toxicity when compared to the wild type gram-negative bacterial pathogen, the method comprising mutating an *htrB* gene within the gram-negative bacterial pathogen, wherein said mutation

causes a phenotype of a resultant *htrB* mutant characterized by endotoxin lacking at least one secondary acyl chain on lipid A contained in the wild type gram-negative bacterial pathogen.

28. A mutant gram-negative bacterial pathogen of substantially reduced toxicity, made according to the method of claim 27, wherein the gram-negative bacterial pathogen having substantially reduced toxicity is used to generate endotoxin-specific antibodies.

29. A method for producing endotoxin-specific antisera for a use selected from the group consisting of in diagnostic assays, and for passive immunization, the method comprises immunizing an individual with a vaccine formulation comprising an active ingredient selected from the group consisting of an *htrB* mutant of a gram-negative bacterial pathogen, endotoxin isolated from the *htrB* mutant of said gram-negative bacterial pathogen, and endotoxin isolated from the *htrB* mutant of said gram-negative bacterial pathogen said endotoxin conjugated to a carrier protein; and collecting antibody produced from said immunized individual; wherein said *htrB* mutant lacks one or more secondary acyl chains of lipid A contained in the wild type gram-negative bacterial pathogen resulting in substantially reduced toxicity when compared to lipid A of the wild type gram-negative bacterial pathogen.

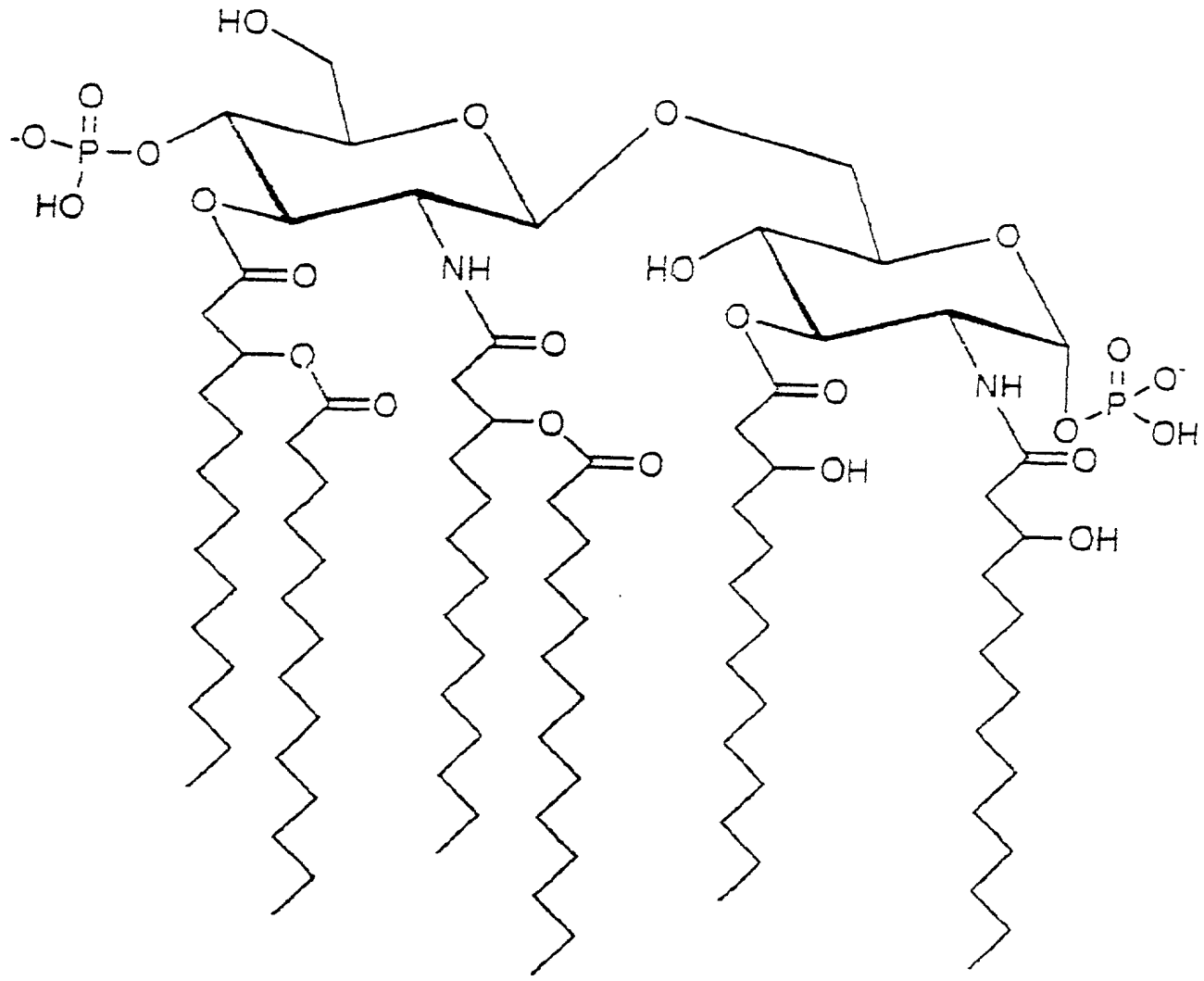
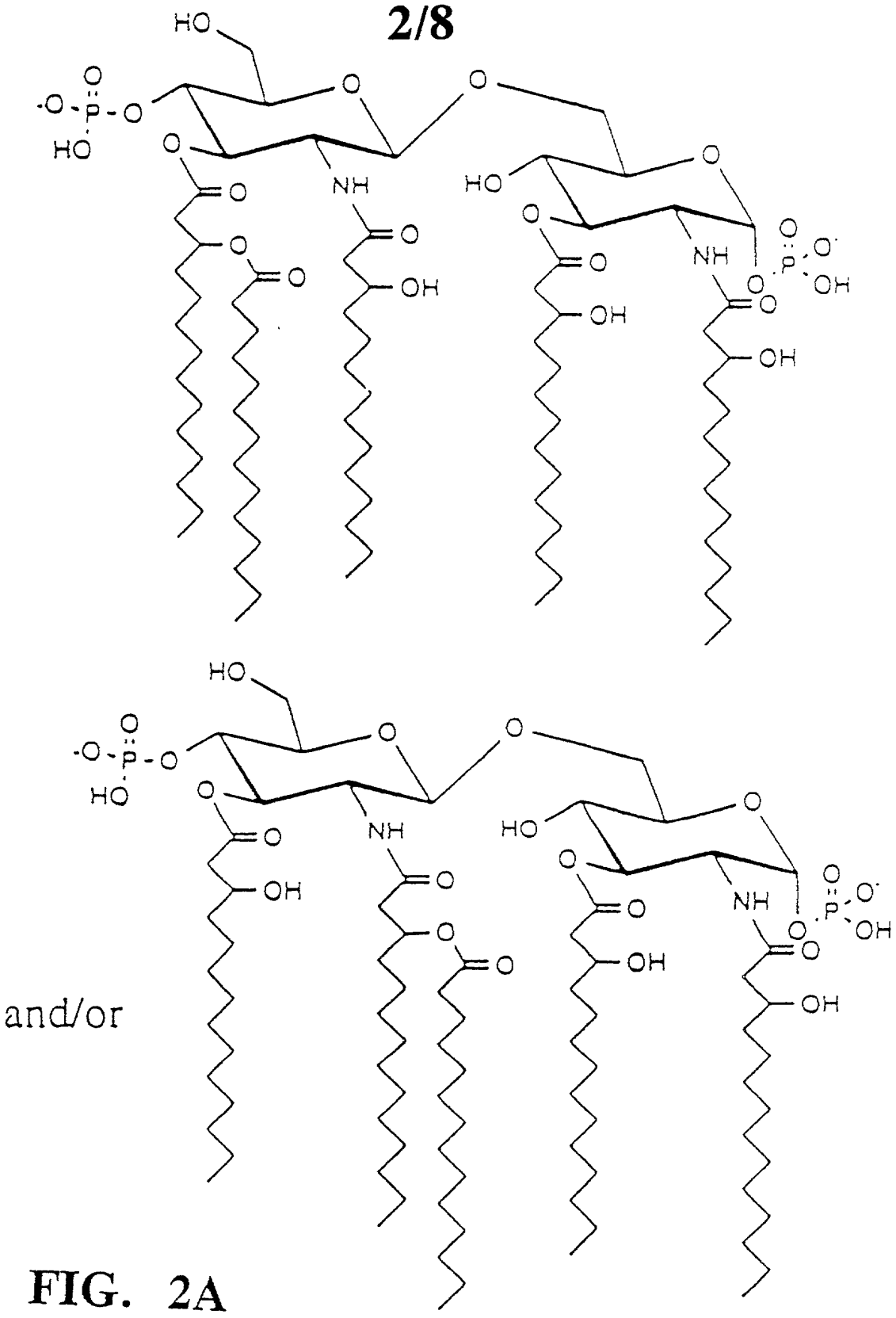


FIG. 1



and/or

FIG. 2A

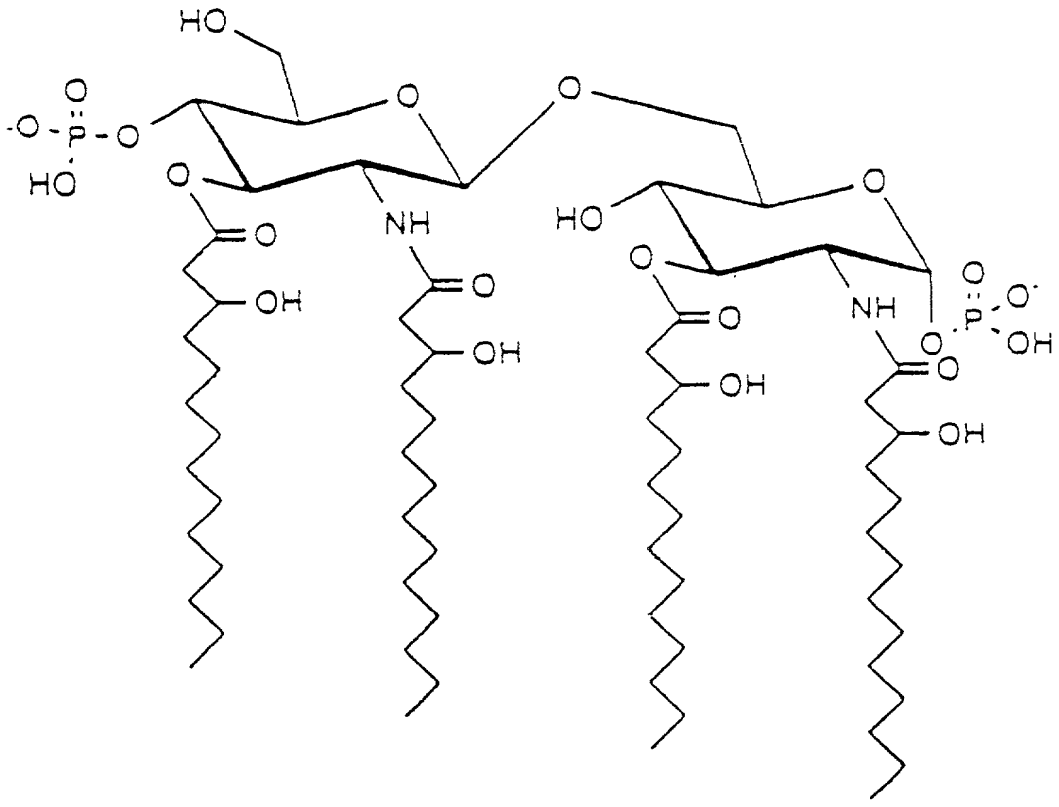
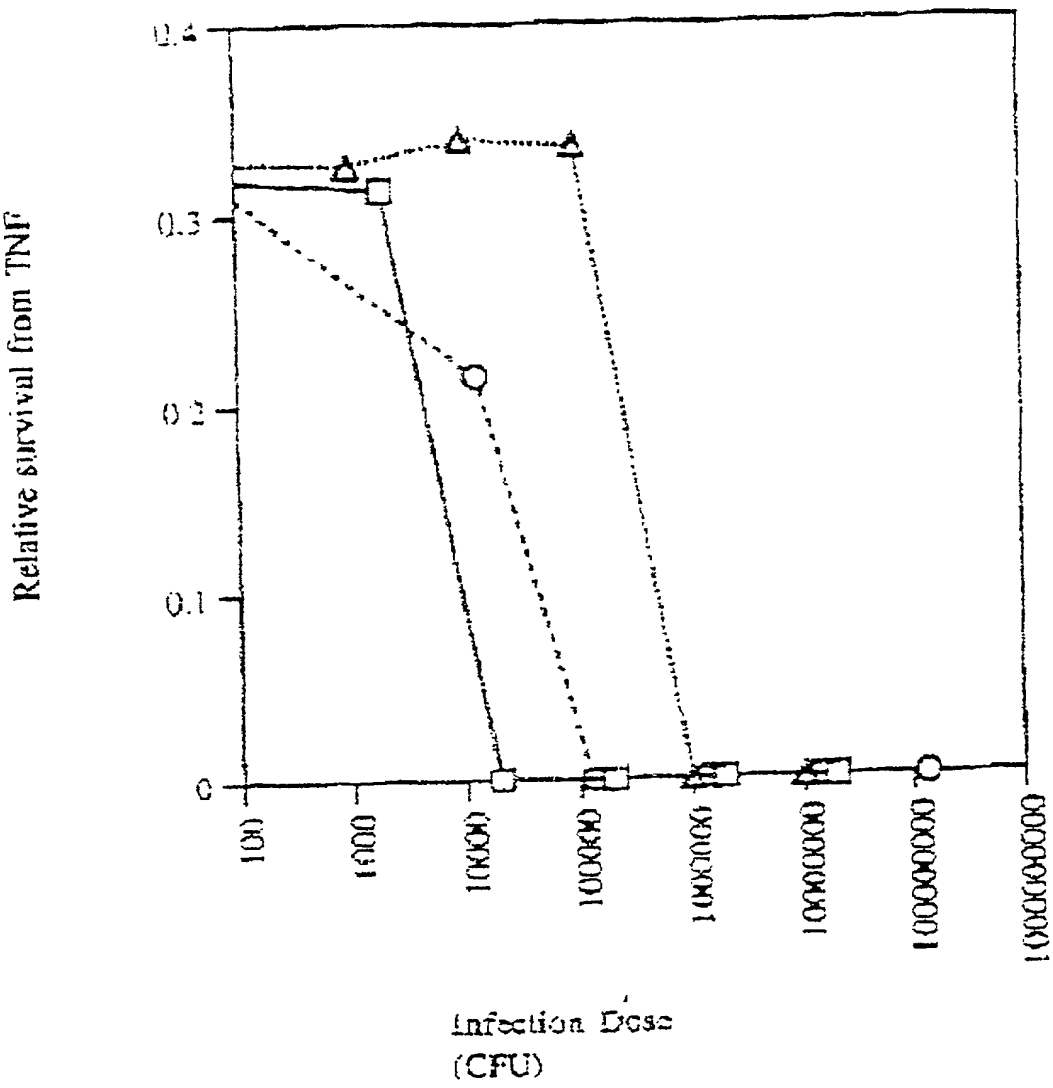


FIG. 2B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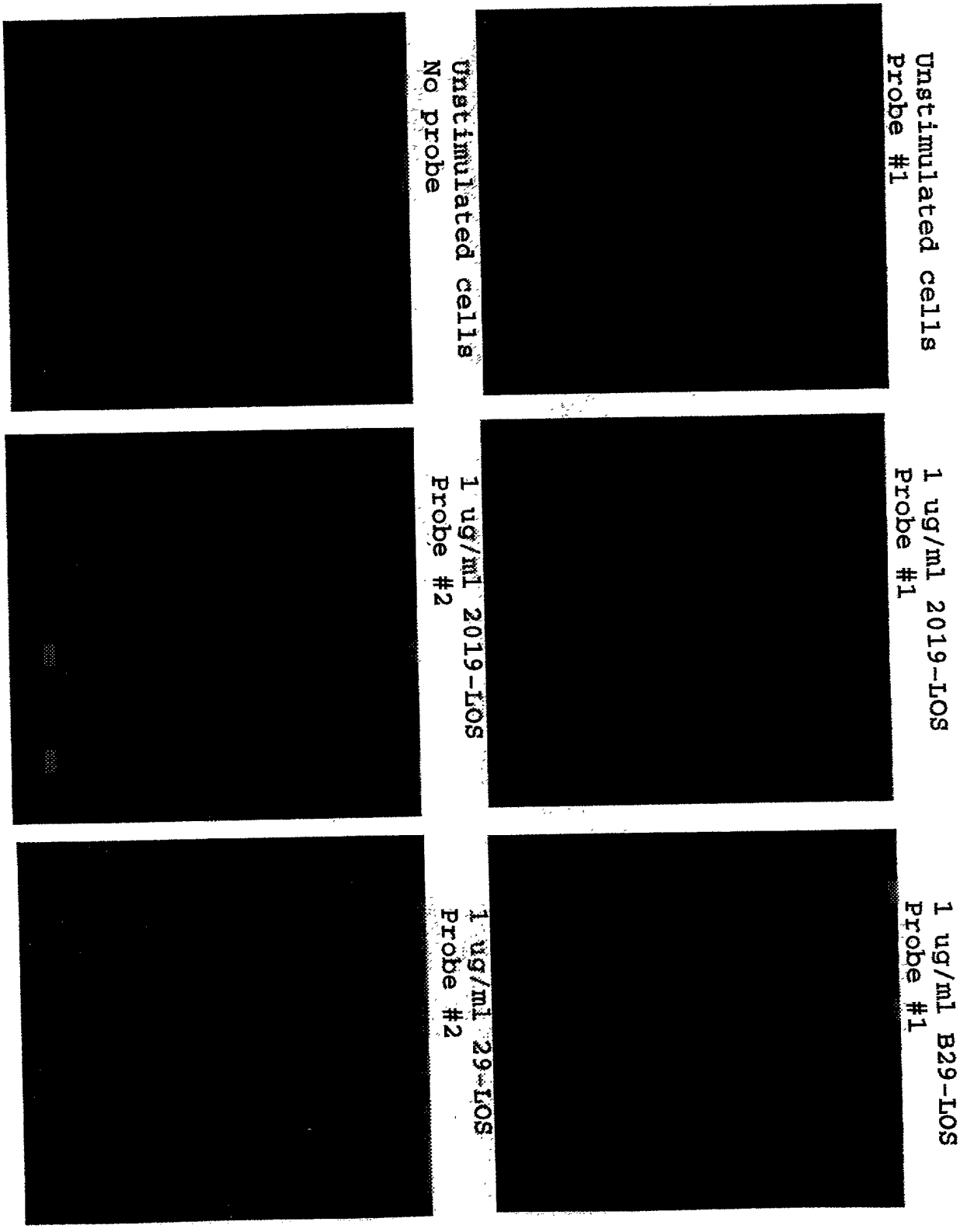
0.3
0.2
0.1
0
100
1000
10000
100000
1000000
10000000
100000000
1000000000
Infection Dose
(CFU)

FIG. 3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Probe #1
1 ug/ml 2019-LOS
Probe #1
1 ug/ml B29-LOS
Probe #1
Unstimulated cells
No probe
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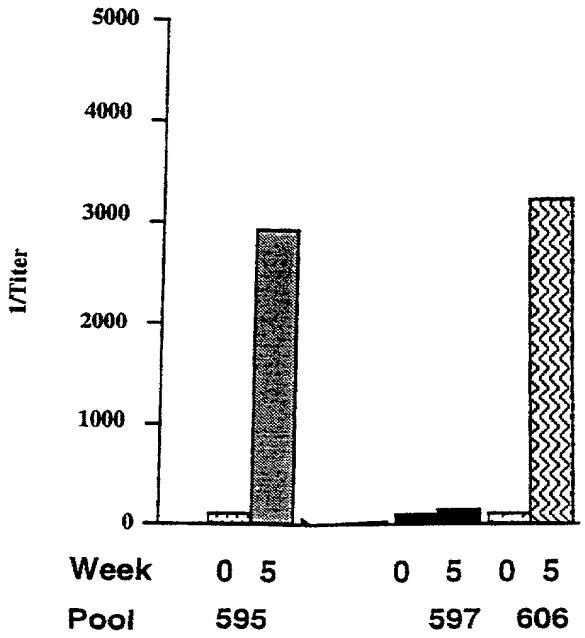
Probe #2
1 ug/ml 29-LOS
Probe #2

FIG. 4


5000
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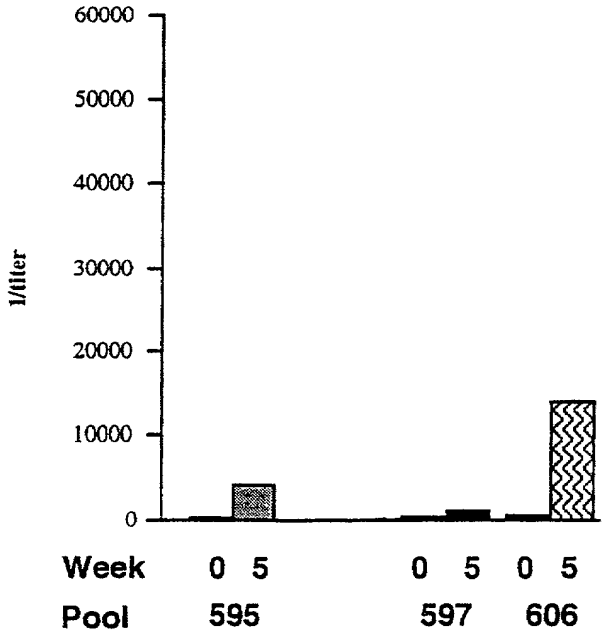
3000
2000
1000
0
1/Titer
Week 0 5 0 5 0 5
Pool 595 597 606

FIG. 5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40000
30000
20000
10000
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Week 0 5 0 5 0 5
Pool 595 597 606

FIG. 6

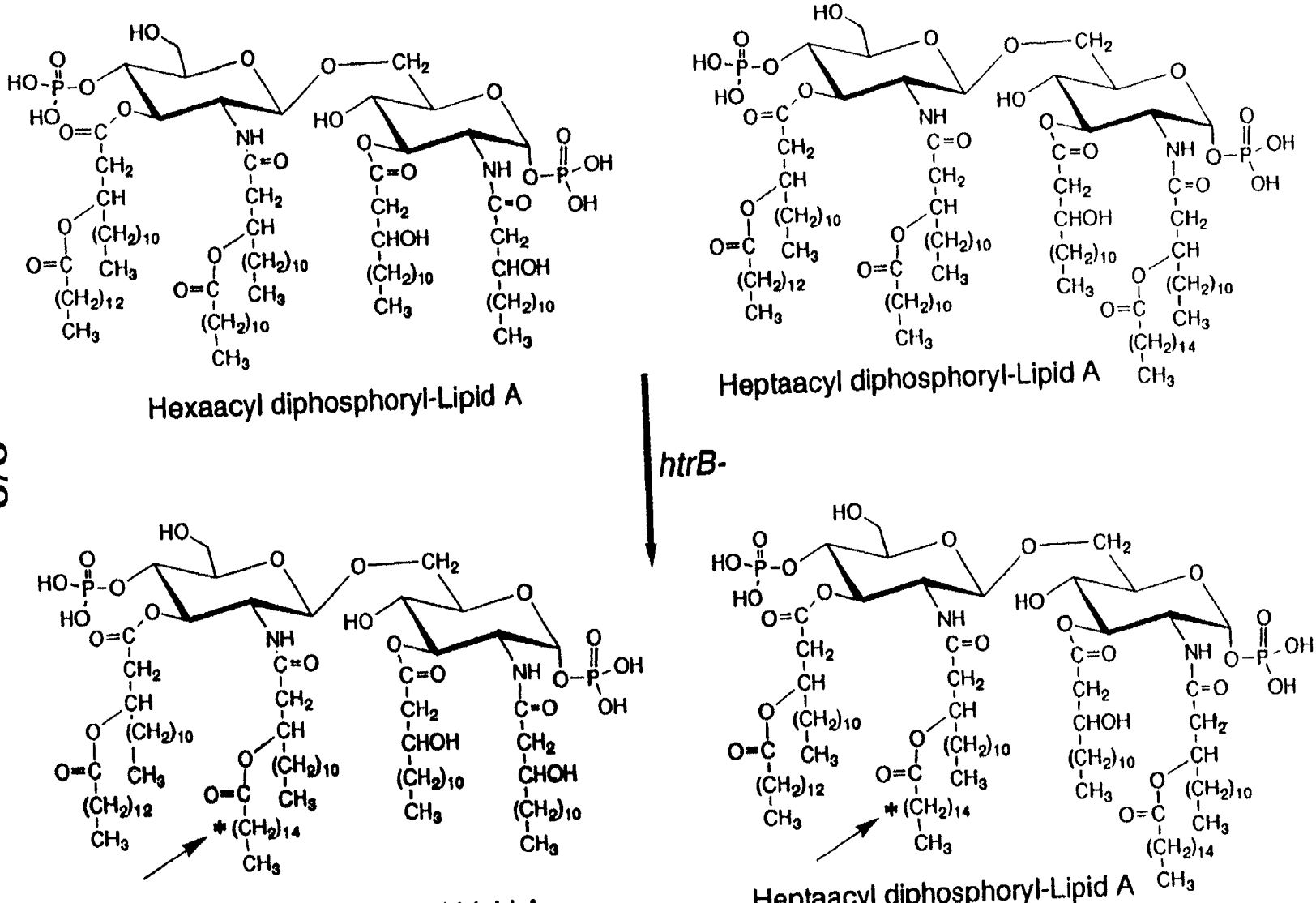

FIG. 7

875-0095

SCHWEGMAN, LUNDBERG, WOESSNER & KLUTH, P.A.

United States Patent Application

COMBINED DECLARATION AND POWER OF ATTORNEY

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Application No.	Filing Date	Status
PCT/US96/18984	November 27, 1996	Complete
08/565,943	December 1, 1995	Pending

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Telephone No. (612)373-6900

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Full Name of joint inventor number 1 : **Michael A. Apicella**
Citizenship: **United States of America** Residence: **Solon, IA**
Post Office Address: 2646 Johnsons Crossing, N.E.
Solon, IA 52333

Signature: _____ Date: _____
Michael A. Apicella

Full Name of joint inventor number 2 : **Melvin G. Sunshine**
Citizenship: **United States of America** Residence: **Iowa City, IA**
Post Office Address: 340 Raven Street
Iowa City, IA 52245

Signature: _____ Date: _____
Melvin G. Sunshine

Full Name of joint inventor number 3 : **Na-Gyong Lee**
Citizenship: **Korea** Residence: **Incheon, Korea**
Post Office Address: Apartment 27-1309
Nam-gu Hakik-2-dong
Sindong-a
Incheon
Korea

Signature: _____ Date: _____
Na-Gyong Lee

Full Name of joint inventor number 4 : **Rasappa Arumugham**
Citizenship: **India** Residence: **Pittsford, NY**
Post Office Address: 15 Elatia Circle
Pittsford, NY 14534

Signature: X _____ Date: July 31, 1998
Rasappa Arumugham

X Additional inventors are being named on separately numbered sheets, attached hereto.

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Full Name of joint inventor number 5 : **Bradford W. Gibson**
Citizenship: **United States of America** Residence: **Berkeley, CA**
Post Office Address: 1324 Peralta Avenue
Berkeley, CA 94702

Signature: _____ Date: _____
Bradford W. Gibson

Full Name of inventor:
Citizenship: Residence:
Post Office Address:

Signature: _____ Date: _____

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Signature: _____ Date: _____

§1.56 Duty to disclose information material to patentability.

(a) A patent by its very nature is affected with a public interest. The public interest is best served, and the most effective patent examination occurs when, at the time an application is being examined, the Office is aware of and evaluates the teachings of all information material to patentability. Each individual associated with the filing and prosecution of a patent application has a duty of candor and good faith in dealing with the Office, which includes a duty to disclose to the Office all information known to that individual to be material to patentability as defined in this section. The duty to disclose information exists with respect to each pending claim until the claim is canceled or withdrawn from consideration, or the application becomes abandoned. Information material to the patentability of a claim that is canceled or withdrawn from consideration need not be submitted if the information is not material to the patentability of any claim remaining under consideration in the application. There is no duty to submit information which is not material to the patentability of any existing claim. The duty to disclose all information known to be material to patentability is deemed to be satisfied if all information known to be material to patentability of any claim issued in a patent was cited by the Office or submitted to the Office in the manner prescribed by §§ 1.97(b)-(d) and 1.98. However, no patent will be granted on an application in connection with which fraud on the Office was practiced or attempted or the duty of disclosure was violated through bad faith or intentional misconduct. The Office encourages applicants to carefully examine:

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(c) Individuals associated with the filing or prosecution of a patent application within the meaning of this section are:

(1) Each inventor named in the application:

(2) Each attorney or agent who prepares or prosecutes the application; and

(3) Every other person who is substantively involved in the preparation or prosecution of the application and who is associated with the inventor, with the assignee or with anyone to whom there is an obligation to assign the application.

(d) Individuals other than the attorney, agent or inventor may comply with this section by disclosing information to the attorney, agent, or inventor.

875.0016US2

SCHWEGMAN, LUNDBERG, WOESSNER & KLUTH, P.A.

United States Patent Application

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Signature: _____ Date: _____
Melvin G. Sunshine

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Korea

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Rasappa Arumugham

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Full Name of joint inventor number 5 : **Bradford W. Gibson**
Citizenship: **United States of America** Residence: **Berkeley, CA**
Post Office Address: 1324 Peralta Avenue
Berkeley, CA 94702

Signature: X _____ Date: X Sept. 16, 1998
Bradford W. Gibson

Full Name of inventor:
Citizenship: Residence:
Post Office Address:

Signature: _____ Date: _____

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retain

SCHWEGMAN, LUNDBERG, WOESSNER & KLUTH, P.A.

United States Patent Application

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Bernkopf, Paul A.	Reg. No. P-41,615	Harris, Robert J.	Reg. No. 37,346	Lundberg, Steven W	Reg. No. 30,568
Bianchi, Timothy E.	Reg. No. 39,610	Hofmann, Rudolph P , Jr.	Reg. No. 38,187	McCrackin, Ann M.	Reg. No. P-42,858
Billion, Richard E.	Reg. No. 32,836	Holloway, Sheryl S	Reg. No 37,850	Polglaze, Daniel J	Reg. No 39,801
Brennan, Thomas F.	Reg. No. 35,075	Huebsch, Joseph C.	Reg. No. P-42,673	Schwegman, Micheal L.	Reg. No. 25,816
Brooks, Edward J., III	Reg. No. 40,925	Kalis, Janal M.	Reg. No. 37,650	Sieffert, Kent J	Reg. No. 41,312
Clark, Barbara J.	Reg. No. 38,107	Klima-Silberg, Catherine I.	Reg. No. 40,052	Slifer, Russell D	Reg. No. 39,838
Drake, Eduardo E.	Reg. No. 40,594	Kluth, Daniel J.	Reg. No. 32,146	Terry, Kathleen R	Reg. No. 31,884
Dryja, Michael A.	Reg. No. 39,662	Lacy, Rodney L.	Reg. No. 41,136	Viksnins, Ann S.	Reg. No. 37,748
Embretson, Janet E.	Reg No. 39,665	Leffert, Thomas W	Reg. No. 40,697	Woessner, Warren D	Reg No. 30,440
Fogg, David N.	Reg. No. 35,138				

I hereby authorize them to act and rely on instructions from and communicate directly with the person/assignee/attorney/firm/organization/who/which first sends/sent this case to them and by whom/which I hereby declare that I have consented after full disclosure to be represented unless/until I instruct Schwegman, Lundberg, Woessner & Kluth, P.A. to the contrary.
Please direct all correspondence in this case to Schwegman, Lundberg, Woessner & Kluth, P.A. at the address indicated below:

P.O. Box 2938, Minneapolis, MN 55402
Telephone No. (612)373-6900

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

1-00

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Signature: Michael A. Apicella Date: July 30, 1998

2-00

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Signature: Melvin G. Sunshine Date: July 30, 1998
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Signature: Na Gyong Lee Date: August 11, 1998
Na-Gyong Lee

4-00

Full Name of joint inventor number 4 : **Rasappa Arumugham**
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Signature: _____ Date: _____
Rasappa Arumugham

X Additional inventors are being named on separately numbered sheets, attached hereto.

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Full Name of joint inventor number 5 : **Bradford W. Gibson**
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Post Office Address: 1324 Peralta Avenue
Berkeley, CA 94702

Signature: _____ Date: _____
Bradford W. Gibson

Full Name of inventor:
Citizenship: Residence:
Post Office Address:

Signature: _____ Date: _____

Full Name of inventor:
Citizenship: Residence:
Post Office Address:

Signature: _____ Date: _____

Full Name of inventor:
Citizenship: Residence:
Post Office Address:

Signature: _____ Date: _____

§ 1.56 Duty to disclose information material to patentability.

(a) A patent by its very nature is affected with a public interest. The public interest is best served, and the most effective patent examination occurs when, at the time an application is being examined, the Office is aware of and evaluates the teachings of all information material to patentability. Each individual associated with the filing and prosecution of a patent application has a duty of candor and good faith in dealing with the Office, which includes a duty to disclose to the Office all information known to that individual to be material to patentability as defined in this section. The duty to disclose information exists with respect to each pending claim until the claim is canceled or withdrawn from consideration, or the application becomes abandoned. Information material to the patentability of a claim that is canceled or withdrawn from consideration need not be submitted if the information is not material to the patentability of any claim remaining under consideration in the application. There is no duty to submit information which is not material to the patentability of any existing claim. The duty to disclose all information known to be material to patentability is deemed to be satisfied if all information known to be material to patentability of any claim issued in a patent was cited by the Office or submitted to the Office in the manner prescribed by §§ 1.97(b)-(d) and 1.98. However, no patent will be granted on an application in connection with which fraud on the Office was practiced or attempted or the duty of disclosure was violated through bad faith or intentional misconduct. The Office encourages applicants to carefully examine:

(1) prior art cited in search reports of a foreign patent office in a counterpart application, and

(2) the closest information over which individuals associated with the filing or prosecution of a patent application believe any pending claim patentably defines, to make sure that any material information contained therein is disclosed to the Office.

(b) Under this section, information is material to patentability when it is not cumulative to information already of record or being made of record in the application, and

(1) It establishes, by itself or in combination with other information, a prima facie case of unpatentability of a claim; or

(2) It refutes, or is inconsistent with, a position the applicant takes in:

(i) Opposing an argument of unpatentability relied on by the Office, or

(ii) Asserting an argument of patentability.

A prima facie case of unpatentability is established when the information compels a conclusion that a claim is unpatentable under the preponderance of evidence, burden-of-proof standard, giving each term in the claim its broadest reasonable construction consistent with the specification, and before any consideration is given to evidence which may be submitted in an attempt to establish a contrary conclusion of patentability.

(c) Individuals associated with the filing or prosecution of a patent application within the meaning of this section are:

(1) Each inventor named in the application:

(2) Each attorney or agent who prepares or prosecutes the application; and

(3) Every other person who is substantively involved in the preparation or prosecution of the application and who is associated with the inventor, with the assignee or with anyone to whom there is an obligation to assign the application.

(d) Individuals other than the attorney, agent or inventor may comply with this section by disclosing information to the attorney, agent, or inventor.